Recent advances in diagnosis of HIV and future prospects

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Received 28 February 2014; revised 06 May 2014; accepted 18 June 2014

Human immunodeficiency virus (HIV) is a major cause of death globally. It causes acquired immunodeficiency syndrome (AIDS) characterized by relentless destruction of human immune system. HIV is a retrovirus that primarily infects the CD4 presenting cells of human immune system, such as, macrophages and dendritic cells. People die of AIDS because the disease remains undetectable for a long time after the initial viral infection. Hence, a simple, accurate and economical technique is required to detect HIV in early stage of infection with high specificity and sensitivity. Presently, HIV is diagnosed by various immunological and molecular techniques, such as, ELISA, rapid tests, Western blot, indirect immunoassay, radioimmuno-precipitation, line immunoassay and nucleic acid based tests. The current methods are non-confirmatory because of certain limitations, which further requires other confirmatory tests. In recent years, research has been focused on alternative methods to improve diagnosis of HIV. HIV biosensor based on specific gene will be rapid, sensitive and specific confirmatory test and can save life of several patients. The present review highlights and updates present technologies and progress in new approaches for the diagnosis of HIV with merits and demerits of present technologies.

Keywords: AIDS, biosensors, ELISA, HIV, HIV diagnosis, RNA sensor

Introduction

Acquired immunodeficiency syndrome (AIDS) is a leading factor of death worldwide. AIDS is a clinical syndrome caused by human immunodeficiency virus (HIV), a retrovirus, which causes broad immunosuppression1-3. The first case of this life threatening disease was identified in 1981 in South Africa4. HIV destroys the cells of body immune system and eradicates the ability to fight against infection5. It is transmitted through unprotected sexual contact with infected partner6,7, contaminated blood transfusion or from infected pregnant women to child8. If infected pregnant women are properly treated with antiretroviral drugs, the chances of infection to baby can be shortened by rate of 1%. A person infected with HIV may not have any symptoms still the virus multiplies and spreads the infection to others. The most common symptom of HIV infection includes diarrhoea, fatigue, fever, headache, mouth sores, body rashes and swollen lymph nodes etc. HIV is classified into HIV-1 and HIV-2. HIV-1 is the cause of worldwide epidemic and it is the most pathogenic strain of the virus. It is categorized into many groups and subtypes, such as, M, N, O and P. HIV-2 is less pathogenic and mostly found in West Africa.

Stages of HIV

Primary Infection

Carrier is a person who is infected with the virus and shows no clinical symptoms but can transmit the infection to others. Sero-conversion of the person is characterized as acute phase or primary phase of HIV infection. Sero-conversion is the point when the infection status of the person starts converting from HIV negative to HIV positive and the body starts producing antibodies against virus. The conversion occurs 4-8 wk after post infection. Sore throat, headache, mild fever, swelling of lymph nodes, oral ulcers and rash are the symptoms shown by 30-60% of individuals at initial stage of infection. Virus replicates very vigorously during the initial stage of infection causing very high viral load. The symptoms usually last in 1-2 wk. At this stage, antiviral therapy can be used to reduce the viral load.
Asymptomatic Stage

This is the silent phase of HIV infection. In this phase, an infected person does not show any clinical symptoms and completely unaware about the presence of virus in the body. A person remains healthy for long time with the normal routine but can infect sex partners. This stage lasts in about 10 yr. HIV level in the blood decreases at very low level, even antibodies are still detectable. Hence, antibody test shows positive results.

Symptomatic Stage

It is more-symptomatic level of HIV infection, which includes lymphadenopathy (abnormality in size, number and consistency of the lymph nodes) and one or more opportunistic infections. The lymph nodes damage due to distractions caused by virus from over 10 yr. HIV frequently mutates and infects more T-cells strongly and the body becomes unable to replace the lost T-cells efficiently. Antiretroviral therapy is used to control the viral infection when CD4 cell count drops to low level. The initial symptoms of viral infection are mild but the continuous destruction of immune system makes it worse. Treatment can prevent the destruction of CD4+ T-cells and support the immunity of the body. This stage is also known as multisystem stage as it infects almost all parts of the body.

AIDS

As the immune system becomes more and more weak, the disease progresses towards AIDS. Patients on the stage of AIDS start developing many numbers of opportunistic infections. These infections develop due to the suppression of immune system and the drugs fail to stop the disease progression. These opportunistic infections are the main cause of death among patients. The criteria of World Health Organisation (WHO) to diagnose the progression of disease towards AIDS differ for both adults and children under the age of 5 years. Young ones are declared AIDS positive when the CD4 count is less than 15% and in the case of children, it is less than 20-25%. The diagnosis may also differ from country to country.

Virology of HIV

HIV is classified under the family Retroviridae and genus Lentivirus. Its genome is single stranded positive sense RNA, which consists of nine functional genes. The integrated form of HIV is known as provirus and its genes are located in the central region of proviral DNA and encodes eleven proteins. Among these eleven proteins, only the Gag, Pol and Env proteins are essential for the infection. The classification of the viral genes is summarized in Table 1. The different forms of proteins are described below:

Table 1—List of HIV proteins and genes

<table>
<thead>
<tr>
<th>Proteins</th>
<th>Genes*</th>
<th>Products</th>
</tr>
</thead>
<tbody>
<tr>
<td>Structural</td>
<td>gag</td>
<td>p7 (nucleocapsid)</td>
</tr>
<tr>
<td></td>
<td>pol</td>
<td>Reverse–transcriptase</td>
</tr>
<tr>
<td></td>
<td>Vpu</td>
<td>Vpu</td>
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<td></td>
<td>vif</td>
<td>Vif</td>
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<tr>
<td></td>
<td>nef</td>
<td>Nef</td>
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<tr>
<td></td>
<td>vpr</td>
<td>Vpr</td>
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</tbody>
</table>


Structural Proteins

**Group Specific Antigen (Gag Protein)**

Gag protein (a precursor of 55 kD) is formed of unspliced viral mRNA. The gag gene encodes the structural protein Gag for the assembly of the complete virus. During the virus maturation Gag protein is processed by protease into MA (matrix protein), CA (capsid protein), SP1 (spacer peptide 1), NC (nucleocapsid protein), SP2 (spacer peptide 2) and p6 protein.

**Gag-Pol Precursor Protein**

The fusion of Gag and Pol proteins led to the formation of viral protease, integrase and reverse transcriptase. During the maturation of the virus, virally encoded asparyl protease separates the Gag-Pol proteins. Pol has RNA dependent and DNA dependent polymerase activity. Therefore, reverse transcriptase converts single stranded RNA (ssRNA) into double stranded DNA (dsDNA). The integrase protein is required for the insertion of proviral DNA into the nucleus of host for further processing.

**Envelope Protein (Env Protein)**

Env protein, a trimer of 160 kD is formed by single spliced RNA. Glycoprotein gp160 is further cleaved
into gp120 and gp 41 by cellular protease. The gp120 attaches to the CD4 receptors of immune cells through specific domains\textsuperscript{18}, whereas gp 41 helps in fusion of viral and cellular membranes, which allows the viral genetic material to enter inside the cytoplasm of newly infected cells\textsuperscript{19,20}.

**Regulatory Proteins**

**HIV Trans-activator (Tat Protein)**

Tat is the transcriptional trans-activator, which is essential for viral replication\textsuperscript{21}. Tat is expressed by fully spliced mRNA, which enhances the elongation phase of HIV transcription to produce the full length transcript\textsuperscript{22-23}. Short transcript is generated in the case of less Tat activity. It also activates the expression of various cellular genes, such as, tumor necrosis factor beta (TNF-\(\beta\))\textsuperscript{24} and transforming growth factor beta (TGF-\(\beta\))\textsuperscript{25,26}. It also decreases the expression of genes, which includes B-cell lymphoma 2 (bcl-2)\textsuperscript{27}, chemokine and macrophage inflammatory proteins-1 alpha (MIP-1\(\alpha\))\textsuperscript{28}.

**Regulator of Expression of Virion Protein (Rev Protein)**

Rev protein consists of 13 kD and is produced by fully spliced mRNA\textsuperscript{29}. Rev binds to the complex RNA structure and forms Rev responsive element (RRE)\textsuperscript{30}. The attachment of RRE and Rev protein assist the export of unspliced viral RNA from nucleus to cytoplasm for the translation process\textsuperscript{31-33}.

**Accessory Proteins**

**Negative Regulatory Factor (Nef Protein)**

Nef is a 29 kD protein and is encoded by single exon. Nef is encoded as early protein of HIV as it accumulates early to detectable level in the cell\textsuperscript{34}. Nef decreases the activity of cell surface CD4 receptors, disrupts the activation of T-cells and stimulates the activity of HIV infection.

**Virus Protein R (Vpr Protein)**

Retrovirus usually infects the cells that undergo mitosis as they require the entry into the nucleus to replicate. HIV is a lentivirus and contains pre-integration complex (PIC), which allows their entry inside the nucleus of non dividing cells\textsuperscript{35-37}. PIC enters the nucleus of virus without affecting its nucleus envelope\textsuperscript{38,39}. Vpr protein is present in the PIC and it plays important role in nuclear localization of pre-integration complex.

**Virus Protein U (Vpu Protein)**

Vpu protein is 16 kD polypeptide, which is located in the internal membrane of the cell\textsuperscript{40}. Vpu down-modulates the CD4 receptors and enhances the release of new virus from the existing one\textsuperscript{41}, whereas in the absence of vpu a huge number of virions can be seen attached on the surface of existing virus\textsuperscript{42}.

**Viral Infectivity Factor (Vif Protein)**

Vif protein (23 kD) is essential for the replication of virus. It degrades the APOBEC (apolipoprotein B mRNA editing enzyme, catalytic polypeptide-like) enzyme released from human body. APOBEC shows anti-retroviral activity by mutating the genetic material of virus\textsuperscript{26}. Vif requires for the replication of HIV in peripheral blood lymphocytes, macrophages and various cell lines\textsuperscript{43}. Virus lacking Vif proteins can enter the cells but cannot be able to synthesize the new virions\textsuperscript{44}.

**Pathogenesis**

HIV virus capsid consists of single stranded RNA and enzymes required for virus replication. Envelope of virus is surrounded by external glycoprotein (gp120) and transmembrane protein (gp 41). The gp120 receptors show very high affinity for the CD4 receptors present in cells, such as, lymphocyte, macrophage and dendritic cells. When the virus binds with CD4 receptor on host cell, the complex undergoes various structural changes\textsuperscript{44-45}. The gp 41 allows the viral enzymes, including integrase, reverse transcriptase (RT) and protease to get released inside the host cell. Entry of RNA inside the cytoplasm leads to activation of RT, which converts RNA into dsDNA (cDNA), followed by integration of this proviral genome into the host genome\textsuperscript{46}. The conversion of RNA into cDNA is always an error prone method, which leads to the development of frequent mutations in the HIV genome and thus causes drug resistance and emergence of many other ailments after therapeutic applications in such cases\textsuperscript{47,48}. cDNA is then integrated into the host genome by using integrase enzyme. During the replication of host genome, the integrated viral DNA is also replicated and transcribed into mRNA, which is further spliced into smaller parts. These spliced parts are then transported into the cytoplasm and translated into functional proteins Tat and Rev. Tat protein is required for efficient transcription and elongation of viral transcripts. Various functional structures are then assembled to give rise a new mature virion, which further infects other healthy cells\textsuperscript{49}.
Current Diagnosis of HIV

The HIV/AIDS was clinically discovered in United States after the death of over half of its sufferers. Death of people occurs because the disease remains undetectable for long time. Rapid detection of disease minimizes illness, disability, death and economic losses. No single detection test is available at present which impact on the lives of victims. The present available methods for HIV detection are either time consuming or not confirmatory on single test. The authentic determination of viral and infected burden is necessary to know the HIV infection, progression of disease and to estimate the efficacy of regimens and vaccines. The review highlights the present and future methods for the diagnosis of HIV infection and their advantages and disadvantages (Table 2). The current methods of diagnosis are described below:

<table>
<thead>
<tr>
<th>Assays*</th>
<th>Advantages</th>
<th>Disadvantages</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serological assays</td>
<td></td>
<td></td>
</tr>
<tr>
<td>A. ELISA*53</td>
<td>Highly automated procedure, therefore screening of large number of sample is possible, easy to perform and cost effective</td>
<td>High range of false positive results</td>
</tr>
<tr>
<td>B. Home HIV test*54-56</td>
<td>Simple technique, which does not require expertise to evaluate</td>
<td>More expensive and less friendly use by patients</td>
</tr>
<tr>
<td>C. Rapid test*59</td>
<td>Provide results earlier as compare to other tests</td>
<td>High range of false positive results</td>
</tr>
<tr>
<td>D. Rapid latex agglutination test*58,62</td>
<td>Requires minimum equipments, take less time (5 min) and can be used where sophisticated laboratory facilities are not available</td>
<td>Non specific results</td>
</tr>
<tr>
<td>E. Dot blot assay*63-64</td>
<td>Simple to perform, which requires less labour and provide sensitivity and specificity equal to ELISA</td>
<td>Highly prone to false positive results</td>
</tr>
<tr>
<td>Molecular assays</td>
<td></td>
<td></td>
</tr>
<tr>
<td>A. PCR*65-68</td>
<td>Rapid procedure with isolation and detection within 4 h, sensitivity and specificity is about 97-98%</td>
<td>Highly prone to false positive results due to cross contamination</td>
</tr>
<tr>
<td>B. P24 antigen assay*69,70</td>
<td>Highly specific as compare to serological assays</td>
<td>Susceptible to false positive reaction because of interfering and immune complexes</td>
</tr>
<tr>
<td>Confirmatory assays</td>
<td></td>
<td></td>
</tr>
<tr>
<td>A. Western assay*71-78</td>
<td>Highly specific and defines the antibodies to specific HIV protein</td>
<td>Expensive, labour intensive and needs expertise to interpret</td>
</tr>
<tr>
<td>B. Indirect immunofluorescence assay*61</td>
<td>Confirmation of the positive test can be interopereated earlier as compare to other methods</td>
<td>Does not provide specific pattern of antigen antibody reaction as western blot. It requires expensive microscope and trained individuals</td>
</tr>
<tr>
<td>Future prospects</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Biosensors</td>
<td>Highly specific, economical, rapid and sensitive</td>
<td>Commercially not available at present</td>
</tr>
</tbody>
</table>

*ELISA: Enzyme-linked immunosorbent assay, HIV: Human immunodeficiency Virus, PCR: Polymerase chain reaction.
patient sample (positive sample), the antibodies get attached to the HIV antigen present in the ELISA plate. A secondary antibody linked with the enzyme is then applied into the plate which binds to the primary antibody. Later, an enzyme specific substrate is applied that causes change in colour or fluorescence. Different ELISA techniques can be used to detect HIV, such as, indirect assay, competitive assay, class specific capture assay and immunometric assay. Advantages of this technique include screening large number of samples at one time. In addition, the sensitivity is >99.5%, easy to perform and also cost-effective. The disadvantage includes high of false positive results.53

Home HIV Test

Home HIV test kit technique was first discovered in 1988 for HIV-1 in pregnant women.54 These kits are available in the market and test can be performed by a finger prick to get a drop of blood sample on a small strip of the filter paper. The dried blood sample remains stable on the filter paper for many days and used for diagnosis of HIV antibodies as well as viral load testing.55-57 The extracted blood sample from dried blood spot is sent to the laboratories where it can be detected by ELISA, Western blot and some other detection methods.58 Home test kit methods are particularly beneficial in remote areas where diagnostic centres are out of reach. The advantage of this test is the requirement of small amount of blood sample, which remains stable at room temp for a month and up to 2 yr at -20°C. However, it is more expensive and test may not be confidential to keep the identity of the person.

Rapid Test

The United States FDA has introduced various rapid tests for detection of HIV. The common rapid tests in use are described below.59

1. Agglutination test uses different types of reactions, which produce clumps or settling patterns in positive cases.
2. Flow through cassettes method detects the HIV antibodies present in the sample when pass through porous membrane and a visible line or dot appear in the case of positive sample.
3. Solid phase test includes dipstick comb assay. HIV antigens are attached on the matrix plate where antibodies present in sample can be detected as a visible dot after applying specific reagents.
4. Immuno-chromatographic strip is a single step method, in which blood sample is combined with a reagent and flow through a porous membrane and positive results are observed by the presence of a line on the membrane.

Rapid Latex Agglutination Test

The latex agglutination test was approved in 1988 by US FDA. The principle of the test is based on agglutination of latex. The agglutination (clumping) test kit consists of a colloidal suspension of latex beads coated with purified viral antigens, such as, gp 41 and gp120 enveloped polypeptide60,63 The kit also contains vials of positive and negative controls with predicted reagents in plastic bottle. Patient samples are applied directly on agglutination card. Interpretation photographs are also included in the test kit. Rapid latex agglutination test requires minimum equipment, takes less time (5 min) and can be used where sophisticated instruments facility are not available.64

Dot Blot Assay

This assay is based on the dot blot technique to detect biomolecules, such as, antibodies. In this assay, specific HIV antigens obtained from the cell culture is placed onto a grid of nitrocellulose paper along with positive and negative control. The detection is carried out by calorimetric reaction on addition of antibody conjugated enzyme and appropriate substrate. The dot blot assay is simple to perform, requires less effort and provides sensitivity and specificity as equal to ELISA.65,66

Molecular Assays

Molecular detection is revolutionizing the clinical practice of infectious diseases. The effects are significant in acute-care where accurate and less time consuming detection assays are required for the treatment of patients. Molecular assays are used to detect the genetic material of the virus in the sample to demonstrate both qualitative and quantitative measurements, which detect the presence of virus as well as amount of viral load. The molecular test can also be used to confirm the results of serological tests.

Polymerase Chain Reaction (PCR)

PCR is one of the most powerful techniques for the amplification of specific DNA sequence. The PCR based technique has revolutionized the detection of infectious diseases. PCR amplicon can be detected
by agarose gel electrophoresis or Real-time-PCR (RT-PCR). In RT-PCR, the detection of DNA/RNA is carried out using fluorescence labelled dye (SYBR green) which binds to double stranded DNA and intensity of fluorescence increases with number of PCR cycle. PCR based diagnosis is rapid (4 h test) and has 97% sensitivity and 98% specificity. However, the test is highly prone to false positive results due to cross contamination.  

**p24 Antigen Capture Assay**

The assay is quite less sensitive compared to RT-PCR, but the presence of p24 antigen in every patients sample boosts the application of this test. p24 antigen is measurable even in the patient with less viral load. HIV p24 antigen capture assay is based on the ELISA with an amendment to detect antigen, not antibody. In this assay, the specific antibody (against to p24 antigen) is attached to the solid phase to capture the antigen present in the sample. After this step, a secondary antibody conjugated with biotin binds with p24 antigen. On addition of streptavidin-peroxidase enzyme, streptavidin binds with biotin and, in the presence of substrate (H$_2$O$_2$) and chromogen (tetramethylbenzidine), produces the colour that is measured spectrophotometrically. The assay is highly susceptible to false-positive results due to interfering immune complex formation.

**Confirmatory Assays**

HIV test cannot be confirmed by single test. Therefore, confirmation of HIV infection is mandatory to perform other tests, which are as follows.

**Western Blot**

It is a highly specific assay for the presence of viral antibodies in the sample. It detects antibodies (against specific HIV antigens) on cellulose strip. Usually, HIV infected cells are ruptured and its proteins (antigen) are separated according to their size in gel electrophoresis. Commercially available Western blot kit contains in-build HIV proteins (antigen), such as, envelope proteins (gp160, gp120, gp41), gag core gene proteins (p55, p24, p17), polymerase chain proteins (p66, p56, p31), on the cellulose strip. After the separation, the proteins are transferred onto nitrocellulose membrane and then patient serum antibody is applied to get attached with specific antigen. Unbounded antibodies are removed and secondary antibody conjugated with peroxidase is added to bind primary antibody. After washing the membrane, coloured band(s) are visualized on membrane strip on addition of substrate (H$_2$O$_2$). The number of bands may vary with patient samples. Positive sample contain at least one viral band of Gag, Pol and Env protein; whereas negative sample does not show any band. The method is highly specific to HIV proteins. The disadvantages of the test are that it is expensive, requires intensive labour and needs expertise to interpret the results. Sometimes, the presence of non specific band (s) on Western blot due to cross reaction with malaria, other retrovirus or group O virus restrict the use of Western blot as diagnostic tool.

**Indirect Immunofluorescence Assay**

The indirect immunofluorescence assay is extensively used for the rapid diagnosis of viral infection by detecting the virus antigen and antibodies. In this technique, HIV infected cells are placed onto the microscopic slide and patient serum (antibody) is added and incubated for few minutes. Unbound antibodies are removed by washing and anti-immunoglobulin antibody conjugated with fluorescence dye are allowed to bind with already present antibody on slide. The fluorescence is examined under fluorescent microscope. The sensitivity of the assay is better than earlier methods for the confirmation the HIV infection. However, it does not provide specific pattern of antigen-antibody reaction as in Western blot. It also requires fluorescence microscope as well as trained technician.

**Line Immunoassay**

Line immunoassay is another confirmatory assay after Western blot and indirect fluorescence assay. In this assay, synthetic peptide antigens are applied on the nitrocellulose paper rather than self prepared antigen as in Western blot. The method is similar as described for other immunoassay test except using synthetic peptide antigen. The use of synthetic antigen reduces the risk of contamination, which leads to less chances of false reactions.

**Future Diagnostics of HIV**

In recent years, advances in the nucleic acid based detection of HIV have been reported by many researchers. The principle of diagnosis is based on molecular interaction between the surface linked oligonucleotides and hybridization with complementary target nucleotides present in the
sample. Biosensor is an upcoming technology and can be widely used for the molecular diagnosis of the diseases. Amperometric electrochemical sensors have already been developed for diagnosis of dreadful diseases like meningitis. The sensor employs ssDNA probe that is immobilized on to the surface of a transducer and behaves as biological recognition element. The analytical devices consist of a biological recognition element, which directly interfaced to a signal transducer. The basic components of modern electroanalytical system for voltammetry are a potentiostat, electrochemical cell and a computer. In recent days, potentiostat and computer are bundled into one package. Biosensors are of great research interest due to high sensitivity and specificity, which may offer a powerful opportunity in early diagnosis and treatment of the diseases. The present method of diagnosis of HIV is under numerous negative factors. They are non-confirmatory, which requires further other confirmatory tests to stamp the presence of infection. Furthermore, these assays are expensive and time consuming. Therefore, there is an urgent need to develop more effective reliable sensing and detection technologies. Biosensor technology offers several benefits over present diagnostic tools, such as, specificity, simplicity, rapidness, continuous monitoring, less expensive and easy in portability of the instrument. The schematic fabrication of RNA sensor is shown in Fig 1.

Conclusions

HIV is the major cause of death and people die because the symptoms of the disease remain undetectable for a long time. Therefore, a simple, accurate and specific method is necessary to detect the infection in early stage to stop the progression of the disease. The current methods for the diagnosis of HIV are non-confirmatory on a single test, which further requires other tests for confirmation of the infection. Due to limitations of the above diagnostic methods, a new rapid and accurate technology for the diagnosis should come into the practice. Hence, diagnosis of HIV disease in early stage of infection through biosensors will open up a new era in the area of molecular diagnostic. Further, it will prove to be more rapid, specific, sensitive and economical, and can save many lives.

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