The application of molecular biology techniques to the nature and treatment of viral diseases

[IMA Mini Project | Group U]

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1. Introduction

The diagnosis and treatment of viral disease were prolonged for many years due to low sensitivity of serology and poor growth of virus in tissue-cultured mediums. The development of nucleic-acid-based amplification and detection methods has revolutionized clinical virology and radically shifted the momentum from the phenotypic expression based cumbersome conventional diagnostic and treatment plans. The high specificity, selectivity, and run around molecular biology techniques coupled with genomics have led to rapid detection of virus, strain characterization, virus loading, resistance detection, and phylogenetic studies of the virus. These techniques are also efficient to conduct the epidemiological potential of viruses and make predictions of new outbreaks.

This research project discussed and reviewed various types of molecular diagnostic techniques used in clinical virology. Following a brief procedure, this paper contains a detailed analysis of the pros and cons associated with the method. We have also compared molecular-based technologies based on the degree of specificity and sensitivity for a particular disease. The potential of these techniques as epidemiological tools is also discussed. It is followed by critically examining the main limitations associated with molecular techniques and corrective measures. After this detailed review, we have reflected upon how techniques demonstrated during IMA laboratory hours and directions given during lecture hours helped understand the viral disease and the molecular methods. Finally, we have discussed the future trends along with the conclusion.

2. Molecular Diagnostic Techniques of Medical Viruses

In these methods, specific nucleic acid sequences are detected, and these nucleic acid-based diagnostic tests can be applied to detect virtually any virus that affects humans.

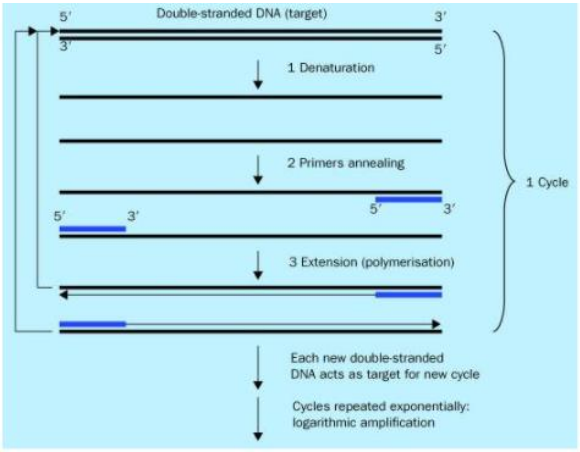
2.1 Nucleic Acid-Based Amplification Techniques:

These molecular techniques are based on the amplification of genomic material. These techniques are extremely sensitive and specific, provide fast diagnosis, and be used to detect several viruses simultaneously.
These techniques help detect viruses that are uncultivable or difficult and harmful to culture, viruses that show antigenic variation and slow-growing viruses in culture. Examples of the most prevalent viruses detected by these techniques are HCV(Hepatitis C virus), HIV, dengue virus, Epstein–Barr virus (EBV), influenza viruses, Zika virus (ZIKV), Ebola virus, and lastly coronavirus. There are several nucleic acid amplification methods currently available for the laboratory diagnosis of viral infections worldwide.

2.1.1 Polymerase Chain Reaction (PCR):

The conventional PCR method is mainly based on thermal cycling. It causes a repeated cycle of heating and cooling in reactants to permit different temperature-dependent reactions such as DNA melting and enzyme-driven DNA replication. PCR employs two reagents - a thermostable DNA polymerase and two specific oligonucleotide primers. PCR is an exponential amplification of the target sequence based on the extraction and purification of DNA molecules. After PCR reaction, the amplified product can be detected by several techniques such as gel electrophoresis, colourimetric methods and sequencing.
PCR has an overall clinical sensitivity ranging from 77.8% to 100% and clinical specificity ranging from 89% to 100%. Conventional PCR is rapidly declining in use because of more advanced techniques like RT-PCR or Real-Time PCR.

PCR can be used to detect most viruses. It has multiplex detection potential. It has several limitations like high risk of contamination, time-consuming, labour-intensive, require thermal cycle and gel documentation apparatus, etc.



Schematic of PCR [Ref: 18]

The PCR reaction takes place in a thermocycler. Each PCR cycle consists of three significant steps: (1) denaturation of template DNA into single-stranded DNA; (2) primers annealing to their complementary target sequences; and (3) extension of primers via DNA polymerization to generate a new copy of the target DNA. At the end of each cycle, the newly synthesized DNA act as new targets for the next cycle. Subsequently, by repeating the cycle multiple times, logarithmic amplification of the target DNA occurs.

2.1.2 Reverse Transcription-PCR (RT-PCR)

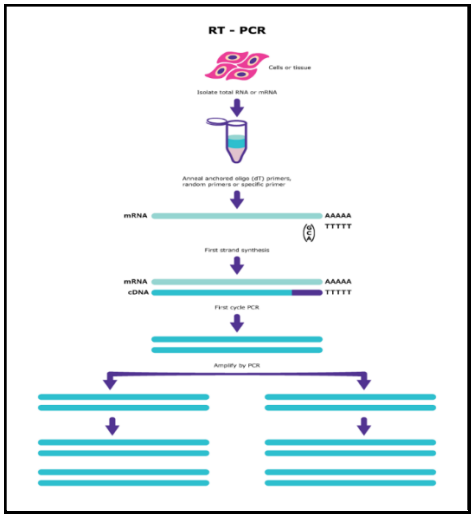
The RT-PCR technique is based on combining reverse transcription of RNA into DNA(in this context called complementary DNA or cDNA) and amplifying specific DNA targets using PCR. It is primarily used to measure the amount of particular RNA. So, it is used primarily to diagnose human infection by RNA viruses.

The use of RT-PCR for the detection of RNA transcript has revolutionized the study of gene expression in the following ways-

1. It has made it theoretically possible to detect the transcript of practically any gene.
2. It has enabled sample amplification and eliminated the need for abundant starting material required when using northern blot analysis.
3. Also, it has provided tolerance for RNA degradation as long as the RNA spanning the Primer is intact.

Conventional RT-PCR demonstrated overall sensitivity ranging from 73% to 100% and specificity ranging from 99% to 100% in detecting viral infection.

It also has several limitations, like RNA handling might be complex, high risk of contamination, time-consuming, expensive, prone to inhibitors.



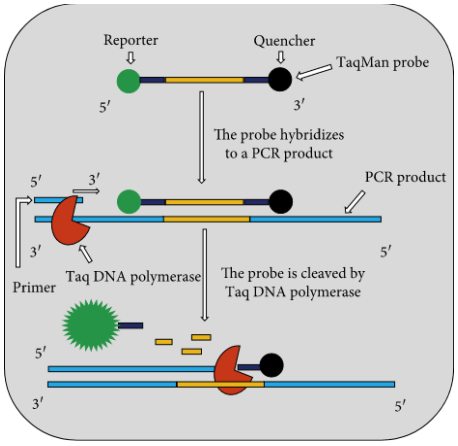
Schematic of RT-PCR [Ref: 19]

2.1.3 Real-time PCR (qPCR)

Real-time PCR is also known as quantitative polymerase chain reaction(qPCR) based on conventional PCR. But it monitors the amplification of a targeted DNA molecule during the PCR(i.e., Real-time), unlike at the end as in conventional PCR. It is a highly sensitive and specific method.

In this technique, the amplification product is detected based on the specimen's amount of fluorescence emission. A unique thermal cycler monitors the emitted fluorescence. The data and the amplification plot is recorded by a computer (connected to the thermal cycler with suitable software) at every reaction cycle.

SYBR green, TaqMan, and molecular beacon chemistries can be used to detect and quantify amplification products. The SYBR green dye binds to the minor groove of double-stranded DNA (ds-DNA) product. Upon excitation by appropriate light, it exhibits an improved fluorescence that is directly proportional to the accumulated dsDNA product.



Schematic of the use of TaqMan probe in qPCR [Ref: 18]

The taqMan probe is a DNA oligonucleotide with two fluorescent dyes termed reporter attached to one end(5' end) and a quencher on the other one(3' end). During the annealing stage, the Primer and the TaqMan probe bind to the template strand. During the extension phase of PCR, the probe is cleaved by 5′ exonuclease activity of Taq polymerase, thereby releasing the reporter from the quencher and resulting in fluorescence emission. The amount of fluorescence is directly proportional to the PCR product.

A molecular beacon is a small DNA molecule with a fluorescent dye at the 5' end and the quencher at the 3' end. The sequences at the 3' and 5' ends are complementary to each other, and the internal part of the molecule is designed to be complementary to the target sequence of a PCR product.

When a molecular beacon is free in solution, it adopts a hairpin structure. This causes the fluorescence and quencher to be nearby, leading to the absorption of fluorescence dye by the quencher, so the fluorescence isn't detected. However, when a molecular beacon hybridizes to the target sequence, the fluorescence and the quencher are separated, leading to the emission of fluorescent light. The amount of fluorescence is directly proportional to the PCR product.

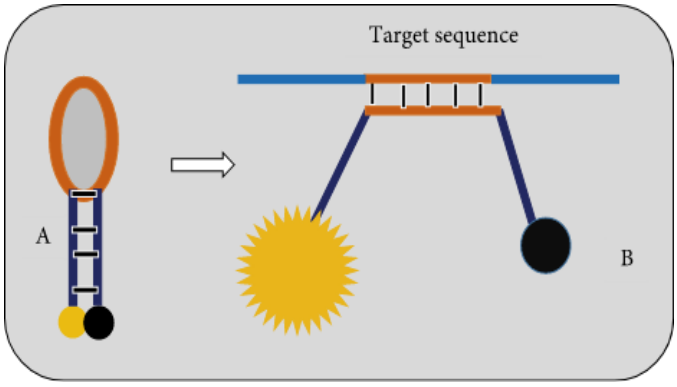


Diagram of molecular beacon [Ref:18]

Diagram of molecular beacon. (A) The molecule forms a hairpin when free in solution. This brings fluorophore (yellow ball) and quencher (black ball) in close proximity, so that no fluorescent light is detected. (B) The molecule hybridizes to the target sequence. This separates the fluorophore and quencher and leads to emission of fluorescent light.

RT-PCR has higher sensitivity and specificity than conventional PCR. It also lowers cross-contamination risk due to tube operation. It is rapid and less labour-intensive, and it can quantitatively determine viral load. There are several limitations to this technique. It is prone to inhibitors like conventional PCR. It also required expensive laboratory equipment and fluorescence probes. Primer dimer artefact is also a problem in the case of the SYBR green method. Also, the designing of TaqMan probes requires almost complete information about the target nucleic acid sequence.

Examples of the use of Real-time PCR in viral testing:

Real-time PCR can be combined with conventional Reverse time PCR to form reverse time quantitative PCR (RT-qPCR). It led to a few advantages over conventional RT-PCR, like reduction of contamination, the possibility of quantifying amplicons and a quick assay time. Due to this, RT-qPCR is widely used in clinical virus specimen such as ZIKV, Ebola virus, coronavirus, HCV, RSV, dengue virus, HIV-1 and influenza A virus. Recently, detection of SARS-CoV-2 is done by RT-qPCR. The assay targeted envelope protein (E) gene and RNA-dependent RNA polymerase (RdRp) gene of SARS-CoV-2. High LoDs of 5.2 copies/reaction for E-gene and 3.8 copies/reaction for RdRp gene were demonstrated, and no cross-reaction with other coronaviruses, suggesting the usefulness of the method for sensitive and specific diagnosis of COVID-19. The RT-qPCR assay, also called Quanty ZEBOV FAST assay was evaluated for
the detection of the Ebola virus in clinical samples. It had an overall sensitivity of 100% and specificity of 98.63%, compared to RealStar Filovirus Screen RT-qPCR Kit 1.0 (Altona Diagnostics).

RealStar® Filovirus Screen RT-PCR Kit 1.0[Ref 13]

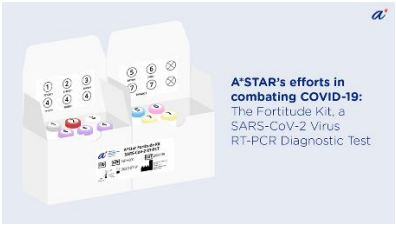
RT-qPCR is also used for diagnosis and monitoring of HIV-1 group O infection with LoD of 40 copies/ml, and specificity of 100% and ZIKV detection in human serum and urine is also applied by this method, and it has LoD of 2.5 PFU/ml and 250 PFY/ml in urine and serum respectively. RT-qPCR is also used for the detection of respiratory viruses in patients in acute respiratory infection when compared its performance with indirect immunofluorescence assay(IFA) and found that RT-qPCR managed to detect viral pathogens in 88(88/162) specimens whereas IFA detected only 33(33/162) specimens.

At present times, there are several RT-qPCR kits available commercially including, the Simplex Dengue RT-PCR assay for detecting and typing of dengue virus serotypes, Real-Star Zika Virus RT-PCR kit 1.0 for detection of ZIKV specific RNA in human serum, Abbott RealTime HCV quantitative assay for HCV RNA quantitation.

Also, the COBAS TaqMan HIV-1 test developed for quantitation of HIV-1 in human plasma. RT-qPCR targets two highly conserved regions of the HIV-1 genome (gag and long terminal repeat). Recently, real-time RT-PCR kits for COVID-19 have been developed. For example, the LOXIN Smart COVID-19 test developed by Co-Diagnostics USA for the detection of qualitative detection of nucleic acid from SARS-CoV-2 in lower respiratory samples and upper respiratory specimens. It has been approved by the US FDA. The assay targets the RdRp gene of SARS-CoV-2. The LoD of the assay is 9.35 ×103 RNA copies/ml with a thermocycler run time of 63–90 minutes, depending on PCR equipment.

A*STAR and Tan Tock Seng Hospital(TTSH) Singapore have developed an RT-qPCR kit(Fortitude kit 2.0) for the qualitative detection of SARS-CoV-2 genetic material in oropharyngeal swabs. The kit has been approved by the Singapore Health Science Authority for clinical use. There are also kits developed by China and a RADI COVID-19 Real-Time PCR kit developed by Korea.

COBAS TaqMan HIV-1 test[Ref 14]

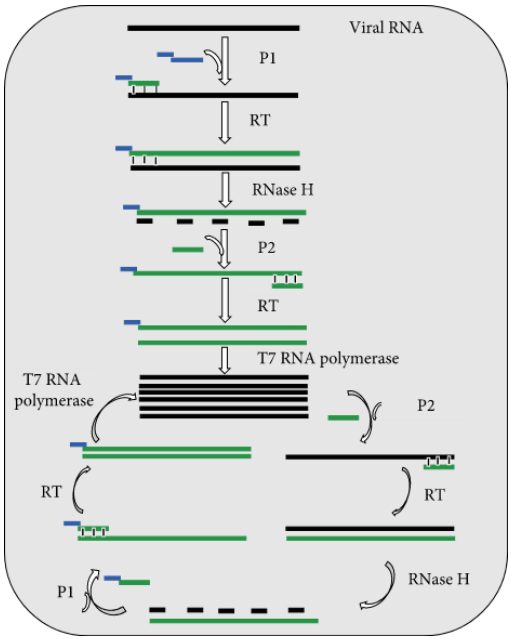


[Ref 15]

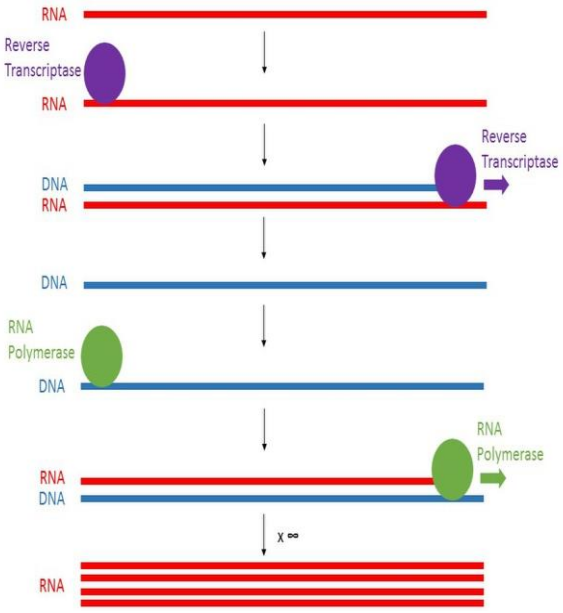
2.1.4 Transcription-Based Amplification Methods

Transcription-Based Amplification methods include two amplifications, namely nucleic acid sequence-based amplification (NASBA) and transcription-mediated amplification (TMA). They are both isothermal amplification methods, and the process is carried out at 41^{o} C temperature. The viral RNA target is converted into cDNA with RT in both cases, and then RNA polymerase synthesizes multiple copies of viral RNA products. The only difference between TMA and NASBA

amplification process is two enzymes, namely RT and RNA polymerase for TMA and for NASBA avian myeloblastosis virus reverse transcriptase (AMV-RT), RNase H, and T7 RNA polymerase. As in the schematic figure of NASBA, It is a two-step process that takes RNA and anneals specially designed primers, then utilizes an enzyme cocktail to amplify it. Similarly, the schematic figure of TMA is also a two-step process that involves the isothermal amplification of rRNA by reverse transcription and subsequent generation of numerous transcripts by RNA polymerase. This method has several advantages. For example, it doesn't require a thermal cycler, so it is affordable, it also has rapid kinetics(requires fewer cycles), and it produces a single-stranded RNA product that is suitable for the detection of various techniques. There are several disadvantages as well. For example, in the case of NASBA, three enzymes are required, has to use enzymes that are not thermostable. RNA handling can be difficult, and the non-specific interaction of the primers may increase as the amplification process occurs at a lower temperature(41^{o} C).



Schematic diagram of NASBA [Ref: 18]



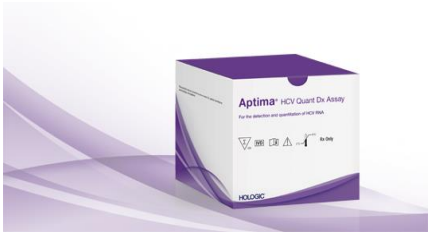
Schematic diagram of TMA [Ref: 20]

Examples of the use of Transcription-Based amplification methods in viral testing

Transcription based amplification methods are suitable for the diagnosis of viral infections caused by RNA viruses. It can multiply viral genomic RNA, messenger RNA and ribosomal RNA. NASBA assay uses gag based molecular beacons to distinguish between HIV-1 subtypes (C and C') circulating in Ethiopia. This assay demonstrated a high level of sensitivity and specificity for both beacons, about 90.5% sensitivity, 100% specificity for the C beacon and 100% sensitivity, 95.2 specificities for the C' beacon by considering sequencing as the gold standard for genotyping NSABA is also used to detect influenza A H5N1 virus in clinical specimens with an LoD of 10 RNA copies/µl along with the same sensitivity as RT-PCR and an average turnaround time of 4 hours. It is also being used for the detection of dengue viral RNA with LoD of 1 PFU/ml for all four dengue serotypes and a turnaround time of 3 hours.

TMA is used for screening blood donations for HIV-1 and HCV RNA. The TMA assay has LoD of 16.2 IU/ml for HIV-1 and 3.5 IU/ml for HCV. Some scientists have used real-time TMA for the detection of HSV-1 and HSV-2 in lesion swab specimens with overall sensitivities of 98.2% and 99.4%, respectively, and specificity of 97.8% and 94.5%, respectively, compared to culture. It was

found in one study that real-time NASBA is more sensitive than conventional RT-PCR in the detection of norovirus.
There are several TMA assays commercially available such as Aptima HCV RNA qualitative assay used for the detection of HCV RNA in human plasma or serum. NASBA based kits are also commercially available for detection, such as The NucliSens Easy Q RSV A and B assay used for the qualitative detection of RSV in respiratory samples of different types.

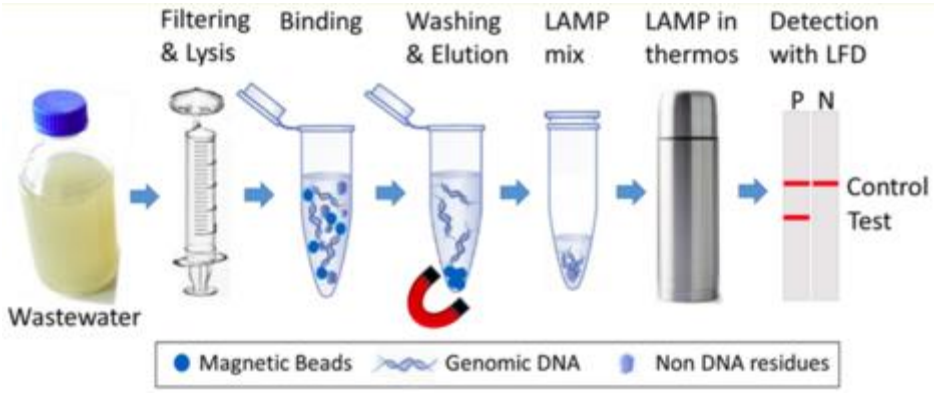


Aptima HCV RNA qualitative assay [Ref 16]

Real-time NASBA Amplification Platform[Ref 17]

2.1.5 Loop-Mediated Isothermal Amplification (LAMP)

LAMP is another isothermal nucleic acid amplification technique. It is a single-tube technique for the amplification of DNA and is extremely used for sensitive, specific, rapid and cost-effective detection of both DNA and RNA viruses in human specimens. In contrast to PCR, this technique is carried out with a series of alternating temperature steps or cycles, so isothermal amplification is carried out at a constant temperature and does not need a thermal cycler.
This method employs 4 to 6 unique primers and DNA polymerase with high strand displacement activity to amplify target DNA. Reverse transcription loop-mediated isothermal amplification (RT-LAMP) combines LAMP with a reverse transcription step to allow the detection of RNA. Primer set for LAMP includes forward inner Primer (FIP), backward inner prime (BIP), forward outer Primer (F3), backward outer Primer (B3). The primers are specially designed to recognize six different regions on the target gene. Then two loop primers (forward loop primer(LF) and backward loop primer(BF)) are also added to accelerate the LAMP assay. Due to these 4 to 6 primers, the LAMP assay has great sensitivity and specificity. The schematics of the LAMP technique is given below.



Schematic diagram of LAMP [Ref: 21]

The method requires only an inexpensive heating block or water bath, making it very useful under poor laboratory settings. The LAMP reaction takes a turnaround time of <1 hour, and the amplified

product can be detected by several methods, including the real-time measurement of the turbidity, visual detection of magnesium pyrophosphate precipitation, detection of fluorescence under ultraviolet light or natural light by adding an intercalating fluorescent dye to the final reaction mixture, and visualization of the bands with various sizes using agarose gel electrophoresis. It has some limitations like the requirement of 6 primers, high risk of carryover contamination, visual detection using the naked eye alone is subjective.

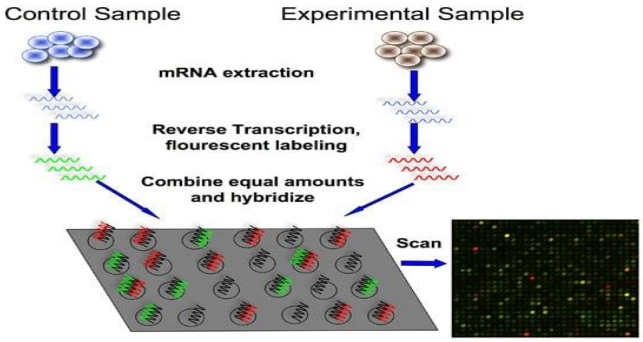
Examples of the use of LAMP in viral testing

LAMP assay has been used for the detection of a number of DNA viruses in human species such as HSV-1 with LoD of 10 copies/µl, hAdV40 and hAdV41 with LoD of between 50 and 100 copies of DNA/reaction and a turnaround time of 1 hour, EBV with the sensitivity of 86.4%, specificity of 100% and 45% of amplification of target sequence and CMV with LoD of 10 DNA copies/µl and turnaround time of 1 hour after RNA extraction. The LAMP method has also been already used for emerging human viral pathogens such as Dengue and SARS viruses. The merging of reverse transcription (RT) into RT-LAMP increases the value of LAMB and allows the rapid detection of RNA viruses in clinical specimens. RT-LAMP assay for the rapid diagnosis of SARS-CoV-2. It is cost-effective, simple, rapid and sensitive. It has also been developed to detect Middle East respiratory syndrome coronavirus (MERS-CoV). The assay has also been developed for rapid detection of dengue virus, influenza A(H1N1) pdm09 virus, H5N1 avian influenza virus, HCV, HIV-1, RSV and ZIKV in clinical samples. RT-LAMP based commercial test kits are available for the detection of SARS-CoV-2, such as the 'ID NOW COVID-19 assay' for direct detection of SARS-CoV-2 in nasal, nasopharyngeal or throat swabs. The assay targets the RdRp gene of SARS-CoV-2. LAMP primer sets such as the Loopamp primer set for avian flu H5 and H7 and FluA influenza are also commercially available.

[Ref: 22]

2.2 DNA Microarrays

A DNA microarray is a collection of microscopic DNA spots attached to a solid surface where each spot contains one or more single-stranded DNA oligonucleotide fragments. It has the capacity to identify medical viruses. A fluorescently labelled target sequences are used to screen an array of oligonucleotide fragments immobilized in a solid surface. The hybridization between immobilized probes and the target sequences results in the detection and quantification of fluorescence-based detection. This technology is a quite high throughput tool because it allows multiplex detection of a large number of potential viral pathogens in clinical samples. It also has several limitations such as too expensive to be used regularly in clinics, labour-intensive and time-consuming.



DNA Microarrays schematics [Ref. 23]

Examples of the use of DNA Microarrays in viral testing

The DNA Microarrays used for high-throughput multiplex detection of viruses in nasopharyngeal samples from children suffering from the respiratory virus. The assay shows an overall sensitivity of 87% to 90% and specificity of =>99% in the detection of RSV, influenza A virus, and rhinovirus/enterovirus compared to RT-PCR. DNA microarrays are also utilized for high-throughput detection of gastrointestinal viruses, viruses transmitted by small mammals and arthropods, herpesviruses, enteroviruses and flaviviruses, HIV-1, HIV-2, and hepatitis viruses and dual infection with two dengue virus serotypes in human specimens. DNA microarray was also used to identify viral causes of meningitis and encephalitis. DNA microarray was also used to detect genotype drug-resistant mutations of HIV, drug-resistant hepatitis B virus mutations, SARS coronavirus and to detect and determine the lineage of influenza B viruses.

2.3 Next-generation Sequencing(NGS)

NGS is very useful in virology detection as it can directly analyze viral fragments extracted from clinical samples. In general, NGS involves three steps- preparation of test samples, sequencing of the target nucleic acid fragments using an NGS platform and third the analysis of the sequencing data. Different companies have produced different NGS machines that use different methods. For example, pyrosequencing (Roche 454) detects the release of pyrophosphate following the incorporation of nucleotides in a DNA polymerase process. Emerging technologies like the Oxford nanopore (MinIon) platform sequences the target nucleic acid by sensing the ionic current of DNA/RNA molecules that pass through the nanopores. Despite its high sequence error rate, Minlon has merits over other NGS platforms because it can generate longer read lengths in real-time, making it suitable for whole-genome sequencing with a short turnaround time, and it is portable, requiring no internet, and also it has a low capital cost. NGS doesn't require prior knowledge of the genomic sequence of the viral pathogens. There are several disadvantages as well, as the turnaround time, number of samples per run, cost of sequencers and requirement of skill in bioinformatics.

Example of use of NGS in viral testing

NGS has been used in diagnostic virology. It has been used for rapid and robust identification of respiratory viruses in clinical samples like influenza A (H1N1) pdm09 virus. It was also used for the detection of HIV-1 drug-resistant mutations. Also, it is used to discover a new Ebola virus.

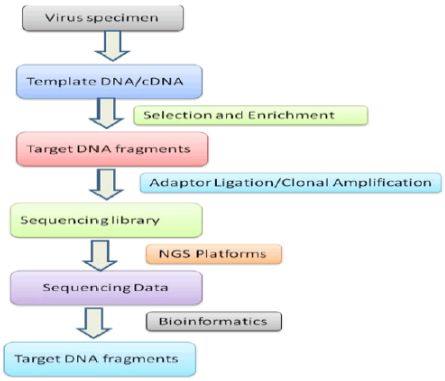


Figure 1. Workflow of next-generation sequencing.

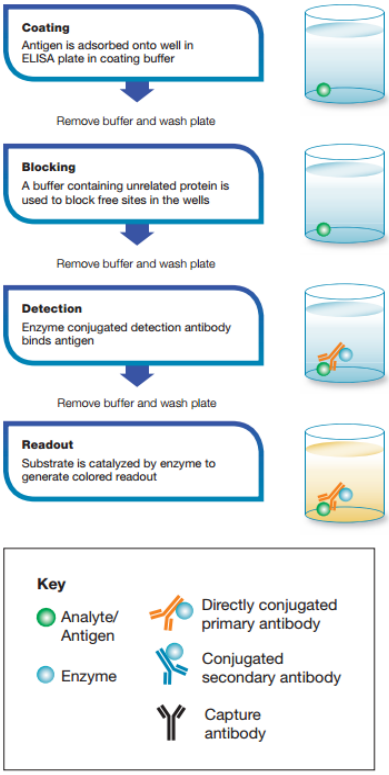
[Ref: 24]

3. Immunological Diagnostic Techniques Of Medical Viruses These techniques are based on the formation of antibodies in response to viral infections. These techniques are simple, high specificity and sensitivity, easy to conduct with multiple samples tested simultaneously. However, these techniques are prone to interference due to cross-reactive agents or endogenous antibodies, leading to false results.

3.1 Enzyme-linked Immunosorbent Assays (ELISA)

This technique is based on primary conjugate antibodies, i.e. pre-attached tags (fluorophore, enzyme or protein) bind to a primary antibody, helping to visualize the specific antiviral antibody or viral antigen in the human system. It is a powerful method to detect and quantify the proteins present in a complex mixture. The positive sample is indicated by the appearance of colour due to the reaction between the primary conjugate antibody and the colourless chromogenic substrate. The intensity of colour obtained is directly proportional to the strength of the antibody-antigen complex. A spectrophotometer measures the colour change. A spectrophotometer determines the absorbance of light by a chemical substance by measuring the intensity of the light passing through the sample. It is performed in 96-well or 384-well polystyrene plates where antibody-antigen interaction takes place. The basic procedure of ELISA involves:

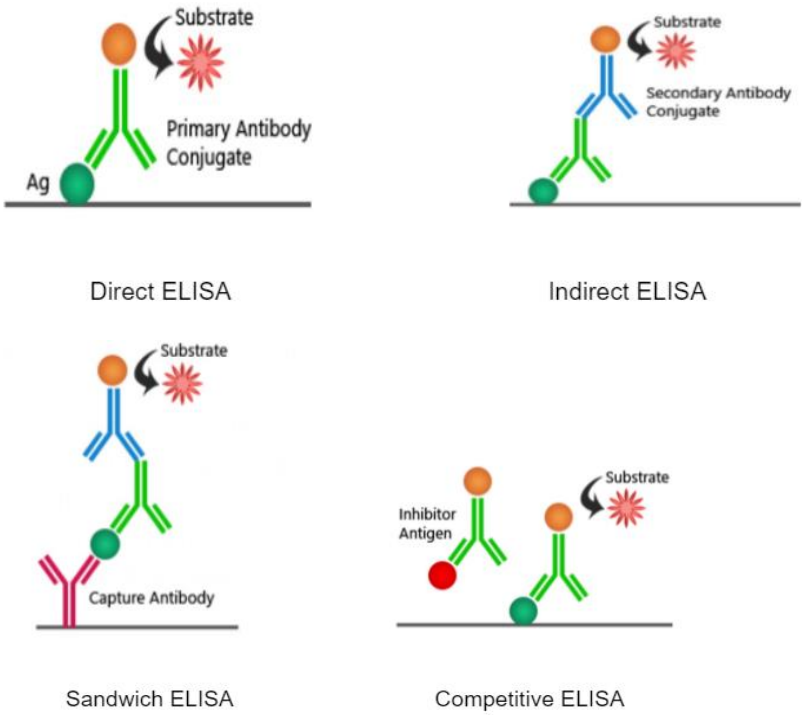
A. Coating/Capture: Immobilization of antigens to the polystyrene microplates.
B. Blocking of the Plate: It involves the addition of blocking buffer irrelevant proteins to saturate the uncovered sites of the microplate.
C. Detection: Binding with antigen-specific antibodies to convert a substrate into a detectable product. Then detection through signals generated by the chemical or radioactive tags associated with the antibody.



ELISA Procedure [Ref: 2]

This makes ELISA a very efficient technique but with a simple and crude procedure. Horseradish peroxidase (HRP), alkaline phosphatase (AP), β-galactosidase, acetylcholinesterase, and catalase are some of the enzymes used commonly in ELISA. The choice of substrate depends on the assay sensitivity and instrument used for the visualization. There are four variants of ELISA in practice.

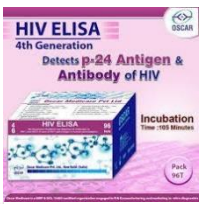
1. Direct ELISA: This detection technique uses a primary antibody labelled with a tag or reporter enzyme directly conjugating with the antigen immobilized on the microplate or via capture format assays. It is generally not used for ELISA but is a frequently used technique in the immunohistochemical staining of cells and tissues.
2. Indirect ELISA: It is the most popular ELISA format in which antigen is detected in two steps. First, an unlabeled primary antibody specific to the antigen binds. Then, an enzyme labelled secondary antibody binds to the primary antibody to amplify the signal. This increases the sensitivity of the technique while sustaining the immunoreactivity of the primary antigen.
3. Sandwich ELISA: In this format, the antigen contains at least two antigenic sites and is sandwiched between detection and capture assays. Both monoclonal and polyclonal antibodies can be used. It removes the need to purify the sample is 2-5 times more sensitive than indirect assays.
4. Competitive ELISA: It measures the concentration of an antigen through signal interference. It is based on the competition between the sample and the reference antigen to bind with the labelled antibody. The more the sample antigen is present, the more will be the binding leading to low detection of reference antigens and hence, the weaker signals.



Different Formats of ELISA [Ref :1]

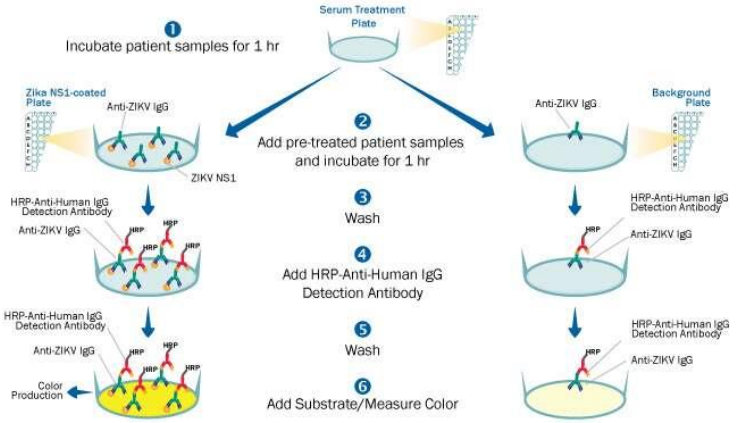
Examples of the use of ELISA for the detection and serosurveillance of human viral pathogens
Antigen-capture ELISA is used to detect MERS-Cov-2 antigens. The assay showed an LoD < 1ng of MERS-CoV-recombinant nucleocapsid protein/ml with a specificity of 100%. It is also used in rapid detection kits for dengue virus NS1 and DENV serotypes with sensitivity and specificity rates of 84.85% and 100%, respectively, in comparison to RT-qPCR diagnostic tests. Anti-ZIKV IgA, IgG or IgM ELISA is used for serodiagnosis of acute ZIKV infections based on ZIKV-1 specific NS1 recombinant antigen. Sandwich ELISA-based commercial kit (HIV 1 and 2 Ag/Ab ELISA kit) uses recombinant HIV antigens (HIV-1 glycoprotein (GP)41, gp120, and HIV-2 gp36) and anti-HIV viral gag protein p24 antibodies to qualitative detect HIV-1 and HIV-2 in human serum samples. Some other medical viruses like Ebola, HSV-2, hepatitis virus and H5N1 influenza virus.

Antibody-Capture ELISA for the detection of SARS-CoV-2 IgM, IgG and IgA in human plasma samples: This technique was tested on 40 RT-PCR confirmed SARS-Cov-2 positive samples with 50 healthy plasma samples as control. It demonstrated 100% specificity and 85% sensitivity as compared to RT-PCR for the detection of IgG and IgM antibodies. In another experiment, conducted with 553 samples from suspected and infected patients, showed 97.9% sensitivity for IgA antibody detection and a specificity >= 96% than immunofluorescence assays. Germany based Euroimmun AG has developed antibody-capture Anti-SARS-Cov2 ELISA IgG kits. S1 protein of SARS-CoV-2 is used as capture antigen in the assay. Epitope Diagnostics, Inc. (USA) has also developed two types of ELISA kits (COVID-19 IgG ELISA and COVID-19 IgM ELISA Kits). The IgG based kit uses SARS-CoV-2 recombinant antigen, and HRP labelled anti-human IgG antibody.



Epitope Diagnostic COVID-19 IgG ELISA kits [Ref :4]

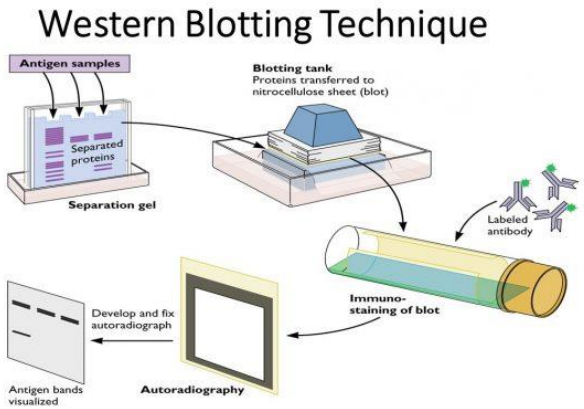
ELISA BASED HIV Kits [Ref :2]



Zika Virus Diagnosis using ELISA [Ref:3]

3.2 Western Blotting Analysis

This technique is used for the diagnosis and analysis of viral proteins and antibodies by separating the denatured proteins through SDS-PAGE. The membrane is blocked to prevent The next step is to electro-transfer the proteins on to a nitrocellulose membrane. Non-specific binding of the antibodies is prevented. It also contains direct and indirect methods. The direct method uses fluorophore/chromogenic substrate-bound primary antibodies. In indirect methods, the transferred protein is then subjected to the primary (specific to virus protein) and secondary antibody (specific to primary protein). Secondary antibody contains chromogenic substrate, which produces a precipitate and is detectable through colourimetry. Fluorescence spectroscopy can be used as an alternative to colourimetry.



The procedure of Western Blotting Analysis [Ref: 5]

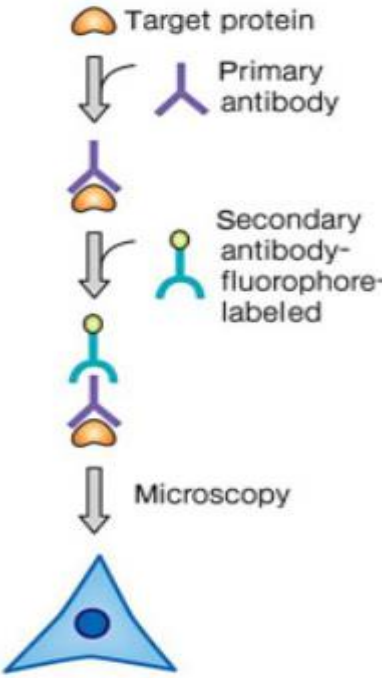
Examples of the use of Western Blotting for the detection and serosurveillance of human viral pathogens

The western blotting analysis is used in the diagnosis of Chikungunya patients. It has shown 83.3% and 96.7 of sensitivity and specificity, respectively, compared to immunofluorescence assays. It can also be a cheap, simple and efficient option for HCV HIV-1 diagnosis in areas with scarce medical resources. Mumbai based J. Mitra and Co. Pvt. Ltd has developed western blot kits for HIV-1 and HIV-2 diagnosis. The kits use nitrogen cellulose membrane strips with HIV-1 viral lysate and HIV-2 antigen (GP 36) with both 100% specificity and sensitivity to licensed western blotting tests.

J. Mitra and Co. Pvt Ltd Western Blot HIV Kits [Ref:6]

3.3 Immunofluorescence Assay

It is the prominently used immunological assay techniques used in clinical diagnosis. Apart from the detection of antiviral antibodies and viral antigens, it is also used to understand the distribution of protein, glycans and other biological macromolecules. Similar to western Blotting and ELISA, it also uses direct and indirect techniques. In the direct fluorescence assay, the fluorophore-conjugated antibody binds to the viral protein. In contrast, indirect fluorescence assays use both primary and secondary antibodies. In indirect fluorescence, the secondary anti-immunoglobulin antibodies bind to each primary antiviral antibody and, thus, is more sensitive. Generally, fluorescein isothiocyanate (FITC) dye is used, and it emits a yellow-green colour. The fluorescence is observed in a fluorescence microscope with the help of UV.



Simplified principle of immunofluorescence [Ref:9]

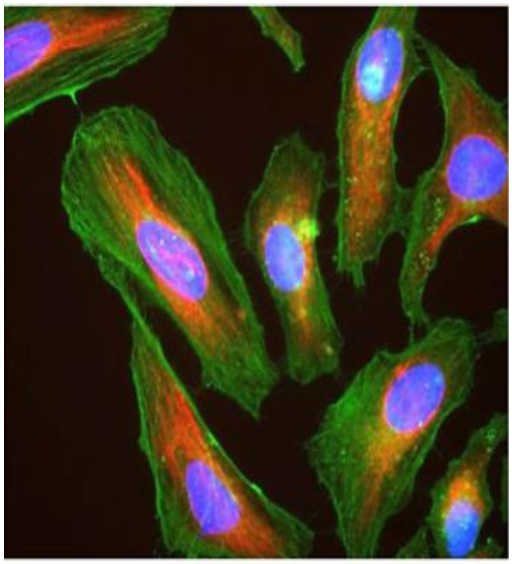


Image obtained under the microscope [Ref: 9]

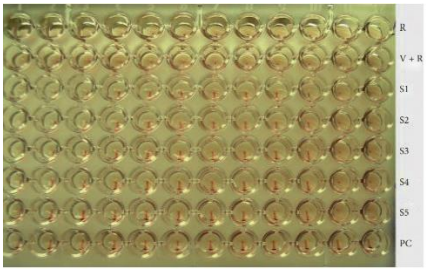
Examples of the use of IFA for the detection and serosurveillance of human viral pathogens

It can detect the presence of SARS-Cov-2 IgG antibodies with a 100% sensitivity and specificity as compared to RT-PCR. Moreover, it can also subtype the influenza A virus with 100% agreement to RT-PCR. It can also be used to test for anti-rabies viral antibodies in human serum with a sensitivity and specificity of 97.2% and 97.9%, respectively, as compared to the mouse neutralization test. It can also be used against HIV as a confirmatory test. IFA based Zika virus kits with ZIKV particles as antigens are available in the market as Anti- ZIKV IIFT, distributed by Euroimmun AG (Germany). This assay demonstrated the specificity of 72.5% and 96.8% sensitivity when evaluated against 126 positive and 102 negative samples. IFA Kits for EBV, HSV are also available.

Indirect Immunofluorescence assay Influenza-A kits [Ref: 11]

3.4 Hemagglutination Inhibition (HI) Assay

The viruses containing envelope protein hemagglutinin can agglutinate RBCs by binding to the cells' sialic acid receptors, forming a lattice. This process is known as hemagglutination. But, if antibodies to the virus are present, hemagglutination is inhibited. In the HI assay, two-fold serial dilutions are done, followed by the addition of viral hemagglutinin and appropriate RBCs to the wells of the plastic tray. So, the absence of hemagglutination means antibodies are produced for the virus, indicating a positive reaction. This is observed by tilting the microtiter plate. The flow of RBC rich serum indicates a positive test. Influenza, dengue virus, adenovirus, rubella and measles are some of the examples.



Hemagglutination assay of Avian Influenza virus. Microtiter plate [Ref: 29]

Examples of the use of HI for the detection and serosurveillance of human viral pathogens

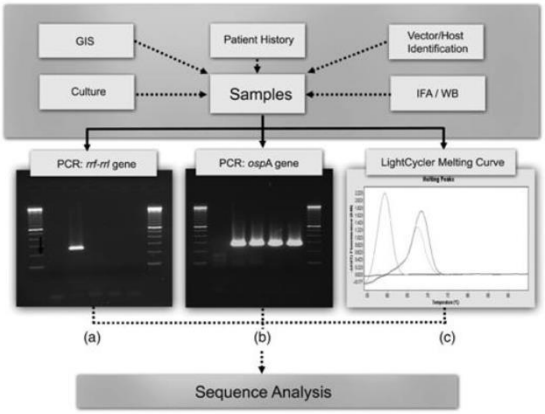
This test is extensively used in the diagnosis of influenza and measles. It was used to test the validity of the vaccine for the H1N1 virus pandemic of 2009. It showed high sensitivity (92%) and specificity (91%) in trials conducted on 79 RT-qPCR-confirmed cases and 176 non-exposed serums.

4. Monitoring Treatment Through Molecular Biology Techniques

There is no specific molecular biology technique directly used for the treatment of viral disease, according to our research. They mostly play a secondary role in the process of treatment, like monitoring of treatment. For example, HIV genotyping for the detection of drug resistance is the standard of care to guide antiretroviral therapy and complements viral load assessment. Genotyping assays have also been seen to be used for disease management, like in patients with Hepatitis C virus (HCV) genotypes other than type 1 respond to interferon therapy. Genotyping assays also have the potential to be used in disease management, checking viral drug resistance and prescribing monotherapy or combination therapy in patients. Also, molecular testing has also been used for the detection of microbial drug resistance. For example, the detection of resistance genes in M.tuberculosis has the potential for clinical application in the near future. This approach has also been used for managing the treatment of Treponema infections. Figure Ref. 11 illustrates the detection of azithromycin resistant *T. pallidum* using PCR-RFLP analysis by detecting a known mutation in the 23S rRNA gene sequence.

5. Molecular Techniques as epidemiological tools

Molecular based strain typing, sequence-based identification, probes to detect genetic markers, DNA fingerprint analysis for heterogeneity etc., has the potential to manage and control diseases by detecting early signs of an outbreak. It can also be used to study the transmission patterns of a virus. For example, the pathogen for Lyme's disease, B. burgdorferi is obtained from biological specimens. Western Blotting, IFA etc., analysis are performed, and confirmatory tests are done by PCR. If the sample shows positive results, tests are performed on the ospA gene and identification is done using the NCBI website's search tool Blast. The identification and other relevant information through phylogenetic analysis to identify multiple strains and their virulence. Another example is syphilis which transmission can be tracked by PCR-restriction frame length polymorphism.



Lyme disease testing: (a) PCR detecting Borrelia species, (b) PCR specified for B. j..Burgdorferi, (c) sequence cluster analysis. [Ref:11]

7. Learnings from IMA

Through IMA laboratory classes on chromatography, we learnt how differential chemical affinity or interaction between different molecules could be used for molecular analysis of a sample. This helped us to understand antigen-antibody interactions in immunological diagnostics. Western Blotting was covered in the class with SDS-PAGE and concepts of gel electrophoresis. This made understanding the need for primary, secondary antibody, transfer of denatured proteins on cellulose membranes easy. IT was fascinating to know how simple methods like derivatization, chromogenic substrate or fluorophore addition for visualization and data interpretation are a central part of medical diagnosis. Prior knowledge of principle, experimentation and nature of applications in the IMA lecture class, we saw important parameters while choosing an instrument for analysis. Here, we saw the practical application of this concept. We understood how an instrument's performance is measured by its sensitivity and specificity from clinical trial samples. But, while designing commercial kits, cost and speed of diagnosis play important roles. Demonstration on interpreting a research paper after each lab experiment came in handy while going through the available literature. Overall, this project introduced us to the commercial and research potential of seemingly basic techniques and principles demonstrated in labs/lectures.

6. Future Prospects and Conclusion

PCR followed by sequencing has become a very popular technique to conduct epidemiological investigations of pathogens. These techniques will soon be commercially available to determine pathogen phylogeny, study of viral life cycles, mechanisms of susceptibility of different host groups, DNA and RNA profile of viral genes etc. Microarray and gene chip assays are widely used in

revealing large amounts of detailed genetic information such as genetic markers of virulence, antibiotic susceptibility and characterization of biosecurity agents. For instance, microarrays are being used to determine protease gene resistance in HIV. It was also used in the sequencing of different species of major variola virus. To eliminate environment contamination of PCR samples, thermal cycling of PCR nucleic acid and fluorimetry are combined into a single sealed container. Viral genotyping and mutation analysis is also supported.
The field of vaccinology is also swiftly observing transition to safer options like recombinant immunogenic proteins, naked DNA vaccines and viral recombinants/mutants. One example is a vaccine against human papillomavirus where genes encoding the proteins of oncogenic serotypes are inserted into baculovirus to produce highly immunogenic proteins. To solve the challenge of weaker immunogenic vaccines, viral vectors are being developed. Recombinant adenoviruses are researched for their potential of viral vectors.
Despite high sensitivity and specificity, these techniques are primarily restricted to research purposes. The commercial use of these techniques is expensive and laborious with concerns of reproducibility. New automated extraction and purification molecular biology techniques with pipetting robots are currently in development. It is highly expected that the new research will give higher output maintaining current testing standards and will reduce the need for expert personnel. The advent of new technology will also raise new questions such as persistence time of DNA after recovery, where did they persist, how to differentiate between colonization and active infection etc.

8.References

1. "ELISA Principles and Types." *Abcam*, www.abcam.com/kits/elisa-principle.
2. “ELISA Kit-HIV 4th Generation.” *Indiamart*, www.indiamart.com/proddetail/elisa-kit-hiv-4th-generation-19256503262.html.
3. “Human Anti-Zika Virus IgG ELISA Kit.” *Rndsystems*, www.rndsystems.com/products/human-anti-zika-virus-igg-elisa-kit_zik00#product-details.
4. “EDI™ Novel Coronavirus COVID-19 ELISA Kits.” *Epitope Diagnostics. Inc*, www.epitopediagnostics.com/covid-19-elisa.
5. “Western Blotting.” *MyBioSource*, www.mybiosource.com/learn/westernblottin.
6. “HIV 1&2 WESTERN BLOT.” *J.Mitra and Co. Pvt. Ltd*, www.jmitra.co.in/services_details.aspx?id=39.
7. DeBiasi RL, Tyler KL. Polymerase Chain Reaction in the Diagnosis and Management of Central Nervous System Infections. *Arch Neurol.* 1999;56(10):1215–1219. doi:10.1001/archneur.56.10.1215
8. Yang, Samuel, and Richard E Rothman. “PCR-based diagnostics for infectious diseases: uses, limitations, and future applications in acute-care settings.” *The Lancet. Infectious diseases* vol. 4,6 (2004): 337-48. doi:10.1016/S1473-3099(04)01044-8
9. “Immunofluorescence Test - an Overview.” *Science Direct*, doi:https://www.sciencedirect.com/topics/medicine-and-dentistry/immunofluorescence-test.
10. “Principle of Immunofluorescence.” *Sino Biological*, ww.sinobiological.com/category/principle-of-if.
11. “INFLUENZA IFA.” *Vircell Microbiologists*, en.vircell.com/products/influenza-ifa/.
12. Muhammad G. Morshed, Min-Kuang Lee, Danielle Jorgensen, Judith L. Isaac-Renton, Molecular methods used in clinical laboratory: prospects and pitfalls, *FEMS Immunology & Medical Microbiology*, Volume 49, Issue 2, March 2007, Pages 184–191, https://doi.org/10.1111/j.1574-695X.2006.00191.x
13. “RealStar® Filovirus Screen RT-PCR Kit 1.0 .” *Altona Diagnostics*, altona-diagnostics.com/en/products/reagents-140/reagents/realstar-real-time-pcr-reagents/realstar-filovirus-rt-pcr-kit-ce.html.

14. "COBAS® TaqMan® HIV-1 Test, v2.0 For Use With The High Pure System." *Diagnostics.roche.com*, ttps://diagnostics.roche.com/global/en/products/params/cobas-taqman-hiv-1-test-v2-0-for-use-withthe-high-pure-system.html.
15. "FIGHTING COVID-19 WITH FORTITUDE." *a-Star.edu.sg*, COVID-19 R&D, www.a-star.edu.sg/News-and-Events/a-star-news/news/covid-19/fighting-covid-19-with-fortitude.
16. " Aptima HCV RNA Qualitative Assay." *HOLOGIC*, www.hologic.ca/products/clinical-diagnostics-blood-screening/assays-and-tests/aptima-hcv-rna-qualitative-assay.
17. "NUCLISENS EASYQ®." *Biomerieux-Diagnostics*, www.biomerieux-diagnostics.com/nuclisens-easyqr.
18. Daniel Hussien Reta, Tesfaye Sisay Tessema, Addis Simachew Ashenef, Adey Feleke Desta, Wajana Lako Labisso, Solomon Tebeje Gizaw, Solomon Mequanente Abay, Daniel Seifu Melka, Fisseha Alemu Reta, "Molecular and Immunological Diagnostic Techniques of Medical Viruses", *International Journal of Microbiology*, vol. 2020, Article ID 8832728, 19 pages, 2020. https://doi.org/10.1155/2020/8832728
19. "RT-PCR — Reverse Transcription PCR." *Merck*, www.sigmaaldrich.com/life-science/molecular-biology/pcr/rt-pcr.html.
20. "Transcription-Mediated Amplification." *Wikipedia*, en.wikipedia.org/wiki/Transcription-mediated_amplification.
21. "Loop-Mediated Isothermal Amplification." *Wikipedia*, en.wikipedia.org/wiki/Loop-mediated_isothermal_amplification.
22. https://embed-fastly.wistia.com/deliveries/6d38da678135882f5f9b75d02dd290ae.webp?image_crop_resized=1280x720
23. "CDNA-Microarray-Experiment." *Bitesizebio.com*, bitesizebio.com/wp-content/uploads/2011/07/cDNA-microarray-experiment.jpg.
24. "Next Generation Sequencing." *Biomedical Research*, www.biomedres.info/articles-images/biomedres-next-generation-sequencing-29-8-1637-g001.png/.
25. Morshed MG, Lee MK, Jorgensen D, Isaac-Renton JL. Molecular methods used in clinical laboratory: prospects and pitfalls. FEMS Immunol Med Microbiol. 2007 Mar;49(2):184-91. doi: 10.1111/j.1574-695X.2006.00191.x. PMID: 17328752.
26. Dwivedi, S., Purohit, P., Misra, R. *et al.* Diseases and Molecular Diagnostics: A Step Closer to Precision Medicine. *Ind J Clin Biochem* **32,** 374–398 (2017). https://doi.org/10.1007/s12291-017-0688-8
27. Speers, David J. "Clinical applications of molecular biology for infectious diseases." *The Clinical biochemist. Reviews* vol. 27,1 (2006): 39-51.
28. Cobo F. Application of molecular diagnostic techniques for viral testing. Open Virol J. 2012;6:104-14. doi: 10.2174/1874357901206010104. Epub 2012 Nov 30. PMID: 23248732; PMCID: PMC3522074.
29. Read SJ, Burnett D, Fink CG Molecular techniques for clinical diagnostic virology *Journal of Clinical Pathology* 2000;**53:**502-506.
30. Reta DH, Tessema TS, Ashenef AS, Desta AF, Labisso WL, Gizaw ST, Abay SM, Melka DS, Reta FA. Molecular and Immunological Diagnostic Techniques of Medical Viruses. Int J Microbiol. 2020 Sep 4;2020:8832728. doi: 10.1155/2020/8832728. PMID: 32908530; PMCID: PMC7474384.