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An Overview of Epidemiology and Diagnostic Techniques for Emerging and Re-emerging Viral Infections

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Abstract

In the past decades, the prevalence of emerging viral infections has escalated and is envisaged to continue to grow in the foreseeable future. More than 17 million people die every year from infectious diseases. The most deadly diseases known to humans so far are caused by emerging and re-emerging viruses such as Influenza, Chikungunya, Ebola, HIV, and the Coronavirus disease 19 outbreak, being the most recent. The clinical prognosis of serious illnesses depends on identification of the infectious agent at the onset. This review outlines the epidemiology and diagnostic techniques used to identify viral pathogens that received particular attention in the recent years. The modern diagnostic tools that are used for identification and confirmation of these disease causing agents such as viral antigen identification, viral culture, nucleic acid analysis, and serology are discussed. While rapid identification of infectious agents, quick diagnosis, and the production of vaccines against a specific virus is possible with advanced laboratory techniques, the limited resources delay the implementation of these techniques. The present need is to understand the importance of early and proper implementation of technological advancements for mitigation of damage caused by infective agents, and the introduction of some novel and appropriate approaches on priority basis in endemic and emerging areas.

Keywords: Viruses, Diagnostic techniques, Nucleic acid amplification, Polymerase chain reaction, Serology

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Introduction

Viruses are submicroscopic infectious agents associated with viral diseases. Viral ailments, such as Acquired immunodeficiency syndrome (AIDS), Severe acute respiratory syndrome SARS, and Ebola, have been the main sources of mortality in people around the globe. While well studied viruses such as HIV continue to kill millions of people, new emerging and re-emerging viruses have become even more troublesome, causing severe outbreak in recent years.[1] The Swine Influenza (H1N1) of 2009, Ebola hemorrhagic fever of 2014, Chikungunya outbreak of 2005, Nipah in 2019 and the coronavirus outbreaks including SARS 2003, MERS 2012, and the most recent Coronavirus disease (COVID) 19 pandemic are among the most devastating infections known to humans so far in terms of human lives lost as well as the huge economic toll.

According to the 2018 World Health Organization (WHO) estimate, 37.9 million people were HIV-infected and 770,000 people died of HIV-related disease in 2018. SARS epidemic of 2003 has affected more than 8000 people and the worldwide financial loss because of SARS outbreak approached the US $40 billion. The Ebola outbreak in 2014 caused 11,310 deaths and the ongoing COVID 19 pandemic has caused unimaginable loss of human life worldwide and unprecedented economic devastation.

To mitigate the severity of infection and transmission, and to avoid and control such epidemic distress, early detection and correct diagnosis of viral infections is imperative. Modern diagnostic procedures involving serological and immunological techniques such as antigen and antibody assessment using serum agglutination, radial immunodiffusion assay, and Enzyme-Linked Immunosorbent Assay (ELISA) can screen large sample sizes in a cost-effective manner within a finite period. More evolved immunological techniques involve ELISPOT assays as well as molecular techniques used for the detection of nucleic acid –RNA and DNA, and involve the use of qualitative and quantitative polymerase chain reaction (PCR) (Real Time PCR). In limited cases, methods such as electron microscopy and fluorescent microscopy are also used to confirm the existence of viral infection.

Fast and accurate diagnosis supports patients by delivering treatment on time, avoiding complications, and improving public health. In a rapid paced environment that incorporates technological advancement, the industry continues to provide up-to-date instruments for diagnosis of viral infections.

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Epidemiology of Viral Infections

According to the WHO, epidemiology is the evaluation of the distribution and determinants of health-related conditions or incidents (including disease), and the application of this research to disease control and other medical conditions. Terms such as epidemic, endemic, and pandemic are commonly used in epidemiological studies; hence, an epidemic refers to the rapid spread of disease to numerous people in a given population above the normal expected levels, whereas endemic refers to the constant occurrence of an infectious disease or agent in a

community within a geographical area.[2] An epidemic becomes a pandemic if it extends to other nations or regions and impacts a large number of people.

Many of the clinical inferences and judgments, including diagnosis, prognosis, and health evaluations, depend directly or indirectly on epidemiological research. Epidemiology has a key role in managing infectious diseases and identifying the origins of cancer, respiratory diseases, and other chronic diseases.[3] It may also help to explain the involvement of viruses in the etiology of diseases, to recognize the association of viruses with environmental factors of disease, to classify factors influencing the vulnerability of the host, to illustrate modes of transmission, and to assess vaccines and therapies on a wide scale.[4] Viral pathogens that have epidemic and pandemic potential are known to cause outbreaks. It is assumed that at least 30 new infectious diseases have emerged over the past 30 years and affected millions of people around the world. The Integrated Disease Surveillance Program network registered a total of 1683 outbreaks of epidemic prone disease during 2017. Statistical analysis found that 71% of such outbreaks were caused by viral pathogens, while 29% were caused by non-viral pathogens.[5]

Moreover, the concept of emergence and re-emergence of a disease plays an important role to understand the etiology of a disease. Emerging infectious disease is the one that has arisen and infected the population for the 1st time, or has occurred before, but is rising exponentially. However, sometimes an old infection reappears in a different clinical type that can also be dangerous or lethal to public health and are referred to as re-emerging infection.[6] There are wide variety of factors contributing to emergence and re-emergence of infection such as mutations of infections agents, human hosts activities and environmental changes, international trade, lack of public health services, and antibiotic resistance.[6,7]

Studies show that most of the agents considered as human pathogens originate from the animal kingdom and 75% of the pathogens responsible for emerging and recurring infectious diseases have the ability to cross animal/human interface. Hence, most of the emerging infectious diseases are zoonotic in nature.[7] Zoonotic infections have been identified among a broad range of human diseases and majority of them arise from domestic, poultry animals, mosquitoes, flies, and also from wildlife species. The mechanism of viral survival and routes of transmission is also an important factor for dissemination of viral diseases. There are three distinct forms of virus survival in mammalian hosts which are characterized by the use of virus reservoirs: (1) Acute self-limiting non reservoir infection, (2) persistent infections with human reservoirs, and (3) the presence of animal reservoir. Out of these most of the viral infections of human are under the group of acute self-limiting infection.[4] The route of transmission of an infection also plays a vital role in understanding the disease etiology. Viral infections can be transmitted by horizontal and vertical forms. Vertical dissemination is used where an infection is spread to the fetus or to an infant in conjunction with delivery during pregnancy. The process by which vertical transmission takes place are: (1) Through the direct incorporation of pro-viral DNA into the Germline DNA of gametes and fertilized eggs, (2) dissemination through placenta during pregnancy, and (3) postnatal transmission through milk feed and other body secretions. Popular examples of vertical transmission are Zika, HIV, cytomegalovirus, rubella, and herpes, etc. Horizontal transmission however is considered the most common mode of transmission and different routes of horizontal transmission of viral infections are explained in **Table 1**.

General and Modern Diagnostic Methods in Virology

The conventional laboratory methods to identify viral infections have been:

1. Direct detection of viral antigen and viral nucleic acid.
2. Virus Isolation,
3. Serology- detection of antibodies in patient's serum.

These serological methods are limited to diagnostic purposes and may incur long waiting periods and be inconclusive in certain circumstances. Direct detection techniques, capable of delivering a conclusive response in <24 h, have experienced significant advancements in recent times.

Virus Isolation

For several years, Virus isolation has been considered the "gold standard" approach for the identification of viral infections. With this method the diagnosis was done by isolating viruses in

Table 1: Horizontal route of transmission of viral infections with example

Route of transmission	*Example*
• Respiratory and oropharyngeal route: By Inhalation of droplets and aerosols emitted by infected person via sneezing, coughing and talking. Touching objects and environmental surfaces contaminate with infected respiratory secretions and this provides a pathway for virus to the nose and mouth of new individual.	Chickenpox, rubella, herpesvirus, cytomegalovirus, Influenza virus, SARS CoV 2, Rhinovirus, Adenovirus, Epstein-Barr virus.
• Gastrointestinal route These viruses are shed in feces and vomiting Infection occurs via ingesting contaminated food and water. Transmission from one person to another via fecal-oral route	Rotavirus, Norovirus, Astroviruses and hepatitis virus.
• Urogenital route Primary Urogenital infections are transmitted through sexual intercourse. Through Urine (Not a major transmission factor)	Human Immunodeficiency Virus, Herpes simplex virus, Human papillomavirus, Hepatitis B virus.
• Cutaneous Route Through unbroken skin, bite of an infected animal and by blood borne routes.	Rabies virus, Poxviruses, HIV, Hepatitis B virus.
• Ophthalmic Route Through patient's fingers, aerosols, infected ophthalmic equipment, etc.	Vaccinia, Herpes simplex, prions, adenovirus, measles.
• Nosocomial transmission Infections that happen as a consequence of a hospital stay or are created by microorganisms and viruses acquired during hospitalization	Chickenpox, Influenza virus, Respiratory syncytial virus.

embryonated chicken cells, tissue culture, laboratory animals, and visual inspection of viral particles in sample through electron microscopy.[8] These approaches are expensive, cumbersome as well as time consuming. There are higher instances of false negatives, lack of sensitivity in cell lines, and chances of transfer of live viruses into workers. However, this approach is now being replaced by new molecular and immunological techniques as modern strategies do not suffer from disadvantages of virus isolation.

Serological Assay

Serological diagnosis is generally dependent on the presence of specific IgM antibodies or a significant increase in specific IgG antibody levels between two consecutive samples collected 7–10 days apart.[9] It is believed that the specific avidity of IgG antibodies is generally low during the initial stage of acute infection; however, it rises during the growth of the response. Serological approaches are helpful in many respects, such as this test allows one to know the etiology of mainstream infection even though no virus or components are found in the sample, to evaluate blood products for the incidence of some chronic infections, immune system determination, and to evaluate the levels of herd immunity and the requirement for prophylactic treatments in organ transplantation.[9] Despite their benefits, serological assays also have a range of drawbacks such as difficult interpretation, and low sensitivity targeted to specific antigen.[9] Serological response is often poor in immunocompromised individuals.[9] The principle behind common serological assays are explained in **Table 2**.[8-11]

Molecular Techniques

The much more commonly used virus diagnostic technique is direct demonstration of viral nucleic acids in clinical samples. Nucleic acid based diagnostic tests identify only the particular virus to which the diagnostic reagent is aimed. These techniques identify unique nucleic acid sequences which can be used for the identification of nearly any virus. In particular, nucleic acid amplification assays are attractive for viruses that are difficult or that grow slowly in culture, and viruses for which antigen detection cannot be applied due to low amount of viral antigen for effective detection.[13] Many nucleic acid based amplification approaches including PCR, nucleic acid sequence-based amplification (NASBA), transcription mediated amplification (TMA), strand displacement amplification (SDA), loop-mediated isothermal amplification (LAMP), rolling circle amplification, helicase dependent amplification, and multiplex ligation-dependent probe amplification have been applied for the detection of viral infections.[14]

The use of amplification techniques to detect, genotype, and quantify viruses has some benefits, for example, high sensitivity, reproductivity, and a broad range of dynamic. These methods use enzyme-mediated processes in which many copies of target nucleic acid are synthesized by the enzymes. Two oligonucleotide primers, which bind to complementary sequences, detect amplification materials. The end result is million copies of the targeted sequence generated.[15]

PCR

The PCR is used to clone a fragment of DNA using readily accessible agents in the laboratory. After each step, there is an increase in the

Table 2: Principle of common serology techniques used in diagnostic virology

Technique	*Principle*
Complement Fixation tests	The test is based on the ability of complement, a group of heat-labile proteins found in the plasma of most warm-blooded organisms, to bind to antigen–antibody complexes. As complexes are found on the surface of the red blood cells, the complement induces their lyses, and can be visualized with an appropriate laboratory setup. The test mainly measure IgG antibodies.
Hemagglutination Inhibition Test	Many viruses adhere to hemagglutinin molecules located on the surface of red blood cells, which can lead to aggregation of red cells under appropriate conditions. Preventing this accumulation through particular antiviral antibodies called Hemagglutination inhibition.
Enzyme immunoassay	Patient serum is incubated with an antigen and the bound antibody is visualized using labeled anti-immunoglobulin antibodies after washing and the bound antibody is detected by an enzyme-dependent color reaction. The sensitivity of the color is directly proportional to the antigen-antibody complex.
Radioimmunoassay	In radioimmunoassay, the label is a radioactive isotope and the bound antibody is accessed in gamma counter.
Immunofluorescence	The procedure uses a fluorescein-labeled antibody to stain tissues with particular virus antigens so that stained cells fluoresce under UV illumination. The assay is performed in two formats: direct immunofluorescence assay (DFA) which recognizes viral antigens and indirect immunofluorescence assay (IFA) which detects antiviral antibody or viral antigens.
Western Blotting	Virus is interrupted, proteins are isolated by gel electrophoresis and blotted to nylon membrane; antibodies in the serum test bind to viral proteins; labeled anti-Ig attaches to individual bands; exposed by EIA or autoradiography.
Neutralizing Antibody Assay	In the assay, known quantities of infectious virus are combined with the serum sample and incubated for a brief period of time after the residual infectivity is assessed using cell cultures or test animals. This infectivity is then compared to the infectivity of the original virus, and the neutralizing potential is determined from this effect.
Latex Tests	These tests involve application of serum or other samples directly on a strip of suitable material such as cellulose, where the antibodies are diffused laterally and eventually reach a site in the strip where appropriate antigen has been applied and chemically fixed. Specific antibodies become bound to the site while non-reacting antibodies diffuse out from the area. The presence of antibodies is visualized using labeled conjugates.

(*Contd*...)

Table 2: (*Continued*)

Technique	*Principle*
Time-resolved fluorescence immunoassay (TR-FIA)	Non-isotope immunoassay in which the indicator antibody is labeled with fluorophore (a europium chelate). The fluorophore emits fluorescence of a different wavelength after excitation by light that can be measured in a fluorometer.
Luciferase immunoprecipitation system (LIPS)	It is a quantitative immunoprecipitation test that uses luciferase-tagged antigens. LIPS have been used for diagnosis, viral discovery and anti-viral monitoring of MERS and HKU2.[12] Luciferases from varied origins, including Gaussia luciferase, Nanoluciferase and Renilla luciferase, have all been used as reporters successfully. Extract containing the recombinant luciferase-tagged viral antigen is used without purification and is incubated with sera/plasma or other antibody-containing fluids. Immune complexes containing antiviral antibodies bound to the viral antigen labeled with luciferase are then immunosuppressed by A/G protein beads and washed. The resultant luciferase activity of the sample shall be measured using a luminometer. LIPS is beneficial in identifying, infected patients from uninfected monitors.

number of DNA molecules and more than 100 billion copies can be quickly identified within a couple of hours.[16] The reaction is based on DNA molecule isolation and purification and exponential target sequence amplification using a thermostable DNA polymerase and two oligonucleotide primers. PCR has been used since its invention to identify human viral infections with a total clinical sensitivity from 77.8% to 100% and clinical accuracy from 89% to 100%.[8] Some of the important modifications of PCR and other molecular techniques are explained in **Table 3** along with their advantages and limitations. The most commonly used variants of classical amplification are real-time PCR and reverse transcriptase PCR (RT-PCR) and they are regarded as gold standard method for viral detection.[1]

Despite its tremendous benefits in virology, its disadvantages include cost-effectiveness, time-consuming and efficiency in the diagnosis of viral infections. The risk of contamination during handling is very high especially during the preparation of the sample.[1]

CRISPR/Cas System-Based Immunodetection

CRISPR/cas9 is a new and unique gene therapy aimed at RNA implemented successfully in 2007 and reported in *Staphylococcus* pneumonia. The CRISPR is RNA sequence repeats that target the foreign DNA cleavage by attaching to the PAM flanking sequences that mediate the endonuclease called Cas through the guide RNA (g-RNA) and are responsible for the host or foreign DNA double strand breaks and silence the gene expression through non-homologous end joining.[25] High analytical sensitivity is achieved using CRISPR effector enzymes such as Cas9, Cas13a or Cas13B, along with a single guide RNA (called sgRNA) which precisely identify the target viral nucleic acid of interest. Gootenberg *et al.* developed a new technology called SHERLOCK (Specific High Sensitivity Enzymatic Reporter UnLOCKing) which combines isothermal pre-amplification with Cas13 to identify single RNA or DNA molecules.[26] Using this technology, author has been able to find ssRNA in Zika and Dengue viruses. Recently, a protocol for identification of SARS-CoV 2 using CRISPR Cas12 system has also been reported.[27]

Microarrays

Microarrays can be differentiated on the basis of attributes such as the design of the probe, the solid-surface support used and the basic approach used to address the probe and/or detect the target. In this technique, oligonucleotide probes bound to solid –surface support and the target is the unknown sequence of interest. The fluorescently labeled target hybridized to the probe microarray. The successful hybridization would lead to an increase in the fluorescence intensity which can be tested via fluorescent detectors.[28] For virus detection, two kinds of microarrays are widely used. In one method short oligonucleotide probes (susceptible to single base mismatch) are used to diagnose or classify known subtypes. However, in the second type, long oligonucleotide probes (60 or 70 BP) are used which allow for sequence mismatches. Microarray technology is an effective method since it scans a wide range of possible pathogens simultaneously.[29] However, the procedure has a range of drawbacks, including being too costly to use for routine clinical diagnostics, time consuming, and labor intensive.[12] Moreover, some novel viruses and divergent strains are difficult to identify with this technology as in microarray approaches; the probes being used have a limited specificity for a specific pathogen. Even, non-specific binding of test substance to hybridization probes can leads to the lack of test sensitivity.[29]

Next Generation Sequencing (NGS)

Next-generation sequencing is a new DNA sequencing method that delivers high speed and efficiency, which can yield large sequence volumes. These platforms have the benefit of evaluating sequence data from single DNA fragments of a library which are isolated into chips, eliminating the need to clone vectors before the sequence acquisition.[30] Preparation of serum, sequencing, and data analysis are the three main steps of NGS. They are classified into short- and long-read methods. Short-read methods are further classified into sequencing by synthesis and sequencing by ligation. Longer sequence readings can be obtained on other applications, such as PacBio, Oxford nanopore and Ion Torrent platforms, which are based on semiconductor technologies (due to a difference in ion charge when a base is applied to the expanding chain).[31] At present, short reading protocols are more efficient and less costly than long reading approaches. The first available short read NGS platform was 454 pyrosequencing (where pyrophosphate released is integrated by a synthesis reaction resulting in the discharge of light), but is constrained by a large error rate.[31] The various NGS platforms use a range of sequencing technologies such as, Illumina sequencers utilize fluorescent synthesis sequencing, ThermoFisher sequencers, and reversible terminators uses semiconductors sequencing, Pacific biosciences using fluorescent nucleotides and Oxford nanopore platform use ionic current sensing.[32] The most recent advancement in this technology is the invention of a cell phone size and portable MinIon platform which has the advantage of being used without internet and external power supply.[31]

Table 3: Important variants of PCR and other nucleic acid amplification methods

Variant	*Principle*	*Advantages*	*Limitations*	*Reference.*
Real time quantitative PCR	Real–time PCR is the standardized way of amplifying and analyzing of nucleic acids at same time without the need of slab gels, radioactivity, and sample manipulation. The PCR material is identified by the use of fluorescent dyes, which ideally bind to double-stranded DNA. The amplification product can be detected by using SYBR green, TaqMan, and molecular beacon.	1). No separate detection methods are required. 2). Low contamination rate 3). High sensitivity 4). May be used for quantitative applications. 5). Multiplex detection 6). Quantitation of viral load.	1). Unable to track amplicon size without device opening, 2). Incompatibility of certain platforms for some fluorogenic chemicals. 3). Start-up costs can be unaffordable when used in low-throughput labs.	8, 13, 15, 17, 18
Reverse Transcriptase-PCR (RT-PCR)	RT-PCR has been developed to amplify RNA targets. This procedure initially generates cDNA from RNA via a reverse transcription and then amplifies the cDNA by PCR.	1). Capacity to identify RNA viruses. 2). Intracellular Signal Analysis. 3). The procedure is quick, easy to use, fast and cost-effective. 4). Capacity to compare different samples together. 4). Can function with limited amount of starting material	1). High sensitivity may leads to false results, even if small amount of DNA is contaminated. 3). Labor and skills intensive.	16, 18
Nested PCR	Two sets of amplification primers and two rounds of PCR are used in this process. The first set of primers is intended to anneal into upstream sequences of the second set of primers that is used in an initial PCR reaction. The first PCR reaction products are used as a template for a second series of primers and a second amplification phase	1). More sensitive and specific	1). High rate of contamination.	15, 19
Multiplexed PCR	In multiplex PCR, sequential amplification of more than one genetic locus is conducted in the same reaction using more than one set of primers.	1). Efficient and cost-effective method for the typing and subtyping of virus strains. 2). Capacity to provide internal controls. 3). Reduce reagent costs.	1). Poor sensitivity or specificity. 2). The existence of more than one primer pair enhances the risk of receiving false amplification products.	13, 16, 20
Nucleic acid-sequence based amplification (NASBA)/ Transcription mediated amplification (TMA).	NASBA and TMA are equivalent to each other. They are strategies for isothermal amplification. At the temperature of 41 ° C, the entire amplification process is carried out. In both cases, the viral RNA target is first converted to cDNA via RT, then several copies of the viral RNA component are synthesized by RNA polymerase. Three enzymes are involved in this reaction: Avian myeloblastosis virus (AMV) reverse transcriptase (RT), RNase H and T7 RNA polymerase.	1). No need of thermal cycler. 2). No denaturation is required prior to detection. 3). Less contamination chances. 4). Faster amplification kinetics.	1). To allow the primers to be annealed to the target, a single melting step is required before the amplification reaction. 2). The temperature of the reaction cannot exceed 42 °C. 3). The range of 120-250 nucleotides should be the length of the amplified RNA target sequence, shorter or longer sequences being amplified less efficiently.	8, 21
Loop mediated isothermal amplification (LAMP)	In this technique, six different primers are designed to identify eight separate regions within the target gene. The amplification continues at constant temperature via strand displacement reaction. Amplification and detection of gene can be achieved in a single step. The procedure consists of three steps: a non-cycling initial step, a cyclic amplification and an elongation step.	1). No thermal cyclers are required. 2). High specificity and amplification efficiency. 3). Rapid, simple and inexpensive 4). both amplification and detection of gene can be completed in a single step 5). Test results can be monitored with naked eyes.	1). Less versatile than PCR 2). Not useful for the purposes of cloning 3). Proper primer designing is required. 4). The greater number of primers per target increases the interaction of the primer-primer	22, 23, 24

In addition to genome sequencing from familiar organisms, it has enabled the invention of novel viruses accounted for unidentified human diseases and the monitoring of outbreaks and pandemics.[1] In a study, 454 NGS technology has shown to be a

potential tool in human papillomavirus (HPV) genotyping.[33] NGS is a good form of typing HPV due to its high sensitivity in multiple infections and its capability for identifying a broad range of HPV types, subtypes, and variants. HIV is perhaps the most sequenced because of AIDS 'national concern as a significant endemic and its high rate of mutation. The uses of NGS in virology are: 1. Metagenomics, 2. Reconstruction of the whole viral genome, 3. Interpretation of the intra-host variability, and 4. HIV antiretroviral drug resistance. However, the use of NGS in clinical laboratories is restricted due to the processing period, sample numbers, sequencer costs, and bioinformatics skills requirements.[12]

Microscopy and Mass Spectrometry (MS) Based Approaches

The high resolution power of electro-microscopy (EM) allows experiments to conduct at nanometer scale and offers direct diagnostic and testing pictures of viruses. One of the most important and widely used techniques to identify and characterize new viruses has been transmission electron microscopy (TEM). Negative staining (NS) and thin section TEM of fixed tissues are two methods which are generally used for this purpose.[34] For over 60 years, NS has been developed as a fast, strong, and universal EM technology. In NS aqueous suspension of biological particles is first collected on carbon-coated grids and then stained with heavy metal salts like as phosphotungstic acid.[35] TEM needs only small volumes of high-value samples and is impartial for RNA or DNA Genomes as a primary stage for pathogen identification.[36] For confirmation, another approach called Immuno EM (IEM) can be conducted on the virus suspension before NS or on ultrathin section.[34] IEM has the same concept as ELISA and has the benefits that it deals with raw serum directly, which ensures that no additional purification of immunoglobulins or conjugation steps is needed. Moreover, antibody consumption is low due to the limited reaction volumes required.[36] A scanning TEM detector in a scanning electron microscope may enhance the accuracy of virus quantification; hence, the combined use of TEM and SEM makes it possible to characterize larger objects. In addition, the fine detail of the viral structure can become evident by Cryo-EM when viral preparations are rapidly frozen. Cryo-EM is suitable for the study of the 3D structure of macromolecular assemblies, and the elucidation of 3D compounds allows explaining their role in living cells.[36]

While this method is particularly useful in the detection of viruses, the expense and operation of electron microscopes, the need of well qualified microscopists and time-consuming testing are some of its disadvantages.[34]

MS

The MS theory is based on transforming the sample into charged particles by method of ionization. These charged particles are isolated and measured by a detector according to their mass-to-charge ratio (m/z). The obtained results are compared with a reference database (library) that exists inside the program and is given as an observational spectrum.[1] Matrix-assisted laser desorption ionization and electrospray (ES) ionization methods are most commonly used in clinical laboratories, because they allow analyte ionization in considerable quantities. The hybrid (RT-PCR/ESI-MS) has been able to classify viral pathogens (acute respiratory virus infections and influenza A viruses) usually for some viruses not detected by basic test procedure, and even provide fast and reliable results in a short time.[25] Surface plasma resonance spectroscopy was developed and it has continued to be a reliable optical biosensor and useful method for the diagnosis of dengue virus E protein as well as for the identification of DENV antigen antibodies.[37]

Covid 19 and its Diagnostic Approach

At the end of December 2019, a novel coronavirus (COVID-19) had first been identified in Wuhan, China, among a group of patients with uncertain cause pneumonia who were epidemiologically related to a wholesale market for seafood and wet animals in Wuhan.[38] The pathogenic agent responsible for this pandemic is SARS-CoV-2. In the past two decades, three epidemic diseases have been caused by coronaviruses, including COVID-19, SARS-CoV, and Middle East respiratory syndrome (MERS).[39] SARS was first reported in November 2002 in Guangdong, China, and more than 8000 human infections with nearly 800 deaths occurred between November 2002 and September 2003, and no confirmed cases of SARS were reported after 2004.[40] MERS coronavirus (MERS-CoV) was first identified in Saudi Arabia in 2012 and there have been over 2494 laboratory-confirmed cases and 858 deaths worldwide since 2012 due to MERS-CoV infection.[41]

According to available statistics, the number of confirmed cases worldwide reached more than 75 million by December 19, 2020, of which 1.67 million had died and 42.6 million had been recovered. It is believed that SARS CoV-2 were closely linked to two bat-originate SARS-like coronaviruses, bat-SL-CoVZC45, and bat-SL-CoVZXC21 and 50% linked to MERS-CoV.[42,43] SARS-CoV-2 is an enveloped positive sense single-stranded RNA (ssRNA) virus ranging from 26 kb to 32 kb in length. The virion of SARS-CoV-2 contains 29 903 nucleotides along with four structural proteins; envelope (E), nucleocapsid (N), membrane (M), and spike (S) proteins.[44] SARS-CoV-2 is in the Group of BetaCoVs and it is verified that SARS-CoV-2 utilizes the same SARS-CoV cell entrance receptor angiotensin conversion enzyme II (ACE2). Work also reveals that 2019-nCoV is not using any other coronavirus receptors, such as aminopeptidase N (APN) and Dipeptidyl-peptidase 4 (DPP4).[45,46]

Diagnosis

The gold standard therapeutic diagnostic tool for COVID-19 is the analysis of viral RNA sequences by nucleic acid amplification tests (NAAT) such as real-time RT-PCR (rRT-PCR) and further verified by next-generation sequencing. The specimens to be collected for diagnosis include nasopharyngeal, oropharyngeal swab, sputum, endotracheal aspirate, or bronchoalveolar lavage. While respiratory samples have the highest yield, the virus can be found in other specimens including stools and blood.[47] The coronavirus isolation in cell culture is not performed in laboratories for clinical therapeutic purposes due to unavailability of permissive cell lines, shortage of commercial antisera to verify the culture, high risk to laboratory personnel, and time for testing.[48] The WHO has published numerous RT-PCR methods for SARS-CoV 2 diagnosis.[49] Two of them are listed in Table 4. A variety of other assays such as CRISPR,[50,51] LAMP[52,53] have been designed to diagnose COVID 19 infection.

Table 4: RT-PCR methods to diagnose SARS CoV-2 infection

Manufacturer	*Gene Target*	*Primer sequence (5′→3′)*	*Probe (5′→3′)*
LKS faculty of medicine school of public health of university of Hong Kong	N, ORF-1b-nsp14	N gene: Forward : TAATCAGACAAGGAACTGATTA Reverse : CGAAGGTGTGACTTCCATG ORF-1b-nsp14: Forward: TGGGGYTTTACRGGTAACCT Reverse: AACRCGCTTAACAAAGCACTC	N gene: -FAM-GCAAATTGTGCAATTTGCGG-TAMRA ORF-1b-nsp14: FAM-TAGTTGTGATGCWATCATGACTAG-TAMRA
National institute of viral disease control and prevention, CDC, China	N, ORF1ab	N gene: Forward: GGGGAACTTCTCCTGCTAGAAT Reverse: CAGACATTTTGCTCTCAAGCTG ORF 1ab: Forward: CCCTGTGGGTTTTACACTTAA Reverse: ACGATTGTGCATCAGCTGA	N gene : FAM-TTGCTGCTGCTTGACAGATT-TAMRA ORF-1ab: FAM-CCGTCTGCGGTATGTGGAAAGGTTATGG-BHQ1

Serology

A number of serological assays to identify immunoglobulins developed during the course of COVID infection have been proposed using IgG and IgM antibodies against the viral spike proteins. These assays are not widely used due to poor sensitivity and specificity.[54] More rigorous research is being undertaken to identify neutralizing antibodies that can be used as reliable markers in epidemiological settings and can also be used as correlates of protection in vaccine studies. Mucosal immunity may also play an important role in providing local immunity to individuals. In depth studies using IgA antibodies to understand its role in controlling spread of infection, especially in asymptomatic individuals have to be carried out.

Viruses Received Special Attention and Their Diagnostic Approach

Herpes Virus

Herpes simplex virus type1 and type 2 are both *Herpesvirale* members, a wide group of double-stranded enveloped DNA viruses. There are presently nine members that are known to cause human infection: Eight with human as its natural host and one transmitted as a zoonotic infection from monkeys that can cause fatal encephalomyelitis in humans.[55]

Diagnosis

Diagnostic techniques for the identification of HSV-1and 2 infections includes detection of viral DNA using PCR, antigens detection by direct immunofluorescence tests using fluorescein-labeled type-specific monoclonal antibodies on smears, or by enzyme immunoassay (EIA) on swabs.[56] A G-specific glycoprotein (IgG) testing for HSV can differentiate amongst HSV-1 and HSV-2 with 98% accuracy.[57] Viral culture is the most regularly utilized strategy for the conclusion The drawback of this technique includes as it takes a long wait for culture results, as long as, 7 days, during which time the disease may has grown further.[58]

Influenza Virus

Influenza viruses consist of a segmented genome with enveloped negative-strand RNA viruses. Family alpha- and beta-influenza virus both possess eight segments of influenza A and B viruses, and the gamma-influenza virus involves influenza C viruses comprising seven segments encoding for nine proteins.[59] Bird flu or Avian influenza viruses {Subtypes: A(H5N1), (H7N9), (H9N2)} in aquatic birds are the universal repository for all documented influenza A subtypes and are possibly the primary cause of human pandemic influenza strains.[60,61] Influenza A viruses are classified on the basis of the presence of surface glycoproteins on the virion that is antigenic in nature. These glycoproteins are characterized as hemagglutinin (HA) and neuraminic acid (NA) surface glycoproteins. So far 18 different subtypes of HA and 11 different NA have been described.[61]

Diagnosis

Testing methods used to identify influenza viruses, including viral isolation, immunofluorescence assay, NAAT, and immunochromatography-based rapid diagnostic assay including RT-PCR and serological studies.[61] In an approach a real-time fluorescent based RT-LAMP assay was developed for influenza virus identification.[62] Moreover, an isothermal nucleic acid based amplification test has been developed to rapidly detect and differentiate between influenza A and B virus in a single test tube and this method identify influenza virus within 20 min.[63]

An advanced serological diagnosis of influenza infection include colorimetric assay called magnetic nano(e)zyme-linked immunosorbent assay in which gold nanoparticles and silica-shelled magnetic nanobeads were pooled to track influenza A virus.[64] Viral culture and RT-PCR are considered as gold standard to identify influenza virus. A new one step real time RT-PCR assay has been designed to detect H9N2 lineage of influenza virus with no cross-reactivity against H1–15 RNA from influenza A, B and other respiratory virus.[65] In a study, a quadruple quantitative RT-PCR assay has been developed to simultaneously detect the presence of H7N9 which further identify highly pathogenic and neuraminidase inhibitors-resistance strains with high efficiency in specificity and sensitivity.[66]

Dengue Virus

Dengue is caused by the virus belongs to *Flaviviridae* family, and there are four distinct, though closely related serotypes of dengue-causing virus (DENV-1, DENV-2, DENV-3, and DENV-4). Dengue infection is transmitted by female mosquitoes mostly of the species *Aedes aegypti* and, to a lesser degree, *Ae. Albopictus*.

Zika, chikungunya, and many other viruses are also transmitted by these mosquitoes. Dengue virus is a spherical virus with a diameter of about 50 nm and contains a single-stranded positive RNA genome of about 10–11 kb. Viral genome encodes for three structural proteins: Membrane protein (M), capsid (C), and envelope (E) glycoprotein and seven non-structural proteins, NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5.[67] The viruses use E protein for receptor binding, erythrocyte hemagglutination, antibody neutralization, and defensive immune response.

Diagnosis

The biomarkers which are targeted for the diagnosis of dengue virus include the virus isolation or identification of viral genomic RNA, analysis of NS1 protein or the identification of virus-specific immunoglobulins: IgM and IgG.[68] Nested RT-PCR assay, using universal dengue primers targeting the C/prM genome region for an initially reverse transcription and multiplication step, followed by a serotype-specific nested PCR amplification have been developed successfully.[69] Kong *et al*. identified TaqMan's real-time one-step RT-PCR method for rapid identification, serotyping and quantification of dengue virus.[70] The protocol developed by *Conceição et at* detects dengue virus serotypes 1, 2, and 3 by real-time PCR using genome located in the 5 UTR regions of the viral genome to generate the real-time PCR primers and probes.[71]

Analysis of NS1 Antigens

A protein found in secreted and soluble form from affected host cells, called non-structural protein 1 (NS1), is known to have diagnostic importance as a viral marker of infection.[72] ELISA performed against NS1 antigen revealed that this antigen was present in high concentration in the sera of patients diagnosed with dengue virus infection in the initial stage of disease.[73] The combination of NS1 detection with IgM and/or IgG detection demonstrated a dramatic improvement in positive diagnosis of dengue.[68] A novel assay to identify dengue virus NS1 antigen with a microfluidic immune-magnetic agglutination approach was evaluated in a recent study.[74] Serological methods for diagnosing dengue infection includes, Plaque reduction neutralization test (PRNT), Hemagglutination inhibition (HI), Complement fixation, IgM and IgG- capture ELISA, dot-blot assay, and indirect immunofluorescent assay.[68,75,76] The prevalence of cross-reactive antigenic determinants shared by all four serotypes of dengue viruses and members of the flavivirus family complicates the dengue virus serological diagnosis.

Virus Isolation

For viral isolation, sample (serum, plasma, or biopsy tissue) should be obtained before the 5th day after the beginning of the disease. Several mammalian cell culture used for the study of dengue virus includes LLCMK2, Vero, BHK21, and Mosquito cell lines such as AP-61, Tra-284, C636, AP64, and CLA-1.[75] Clear detection and diagnosis of the virus are mostly accompanied by immunofluorescence assays using serotype-specific monoclonal anti-dengue antibodies.

Chikungunya Virus (CHIKV)

CHIKV is a mosquito born virus that belongs to the Togoviridae family genus alpha-virus**.** In a sylvatic cycle, the virus usually circulates among non- human primates or mammalian reservoirs host, and *Aedes*- species mosquitoes.[77] CHIKV has monopartite, liner, and positive single stranded RNA genome of 11-12Kb. The genome is capped and polyadenylated and contains two open reading frames that encode four non-structural proteins (nsP1, nsP2, nsP3, and nsP4) at 5'end and five structural proteins (C, E3, E2,6 K, and E1) at 3 ' end of ORF.[78] The nsP1 exhibits RNA capping activity, NsP2 functions as a protease and helicase enzyme, NsP3 is essential for the formation of genomes, and nsP4 is RNA-dependent RNA polymerase.[78]

Diagnosis

Virus isolation, molecular detection, and serological tests are the primary laboratory methods used to diagnose infection of the CHIKV.[79] Intracerebral inoculation of suckling mice and a number of cell cultures, such as mosquito (C6/36) and monkey kidney (Vero), cell lines may isolate the virus. Results can take 1–2 weeks and Virus isolation must be performed in biosafety level 3 (BSL-3) laboratories only.[79] Numerous molecular assays have been published to identify Chikungunya infection which includes real-time RT-PCR, conventional RT-PCR isothermal method and multiplex assay**.** Edwards *et al.* 2007 designed a real-time RT-PCR test to detect CHIKV and was designed to detect documented strains of virus and also other genotypes.[80] Nucleotide sequence of part of the CHIKV E1 gene was used to explore the relatedness of the sample. Another approach include Reverse transcription LAMP assay followed by MinION sequencing.[81] A pentaplex rRT-PCR assay (CII-ArboViroPlex rRT-PCR) was established for the identification of specific and sensitive genotypes of ZIKV, CHIKV, WNV, and DENV and the housekeeping gene as internal control in single reaction.[82]

The most common serological techniques used to diagnose CHIKV infection are ELISA and indirect immunofluorescence assays (IFA).[83] IgM-capture ELISA (MAC-ELISA) is commonly employed to detect specific CHIKV IgM. The PRNT may be used to confirm the infection of CHIKV.

Zika Virus (ZIKV)

ZIKV belongs to genus Flavivirus of family *Flaviviridae* and is spread mainly by *A. aegypti*. The name Zika is derived from the Zika forest in Uganda, where this is being detected from the Rhesus monkey in 1947.[84] The structure of this virus is similar to other Flavivirus pathogens such as dengue and west nile virus. ZIKV is classified into two major phylogenetic genealogies, Asian and African.[85] In 1953, human disease caused by Zika was first recognized in Nigeria and then in 2007, a major outbreak was seen on islands of Yap, Micronesia which affected 73% of residents.[86]

Diagnosis

Diagnostic testing for ZIKV infection includes NAAT and serological tests. ZIKV can be detected in different clinical samples such as blood, urine, saliva, CSF, semen, breast milk, amniotic fluid, vaginal secretion, and tissues.[87] Mishra *et al.* designed a one-step RT-PCR real-time pentaplex assay (CII-ArboViroPlexrRT-PCR) for the differential diagnosis of both ZIKV strains, CHIKV, WNV, and all four DENV serotypes.[82] For this analysis, ZIKV strain (PRVABC59) has been cultured. Faye *et al.* designed a one-step RT-PCR assay for detection of ZIKV in human serum and L-15 medium.[88] A RT-PCR assay for identification of ZIKV in human semen targeting antisense

Zika virus RNA has also been designed.[89] The existing serological diagnosis of ZIKV infection is generally based on IgM-capture ELISA, which is concerned with cross-reactivity error between different Flaviviruses. In 2017, a multiplex microsphere immunoassay has been designed to diagnose ZIKV infection.[90] The broadly utilized analytic procedure for ZIKV finding depends on the virus molecular identification by conventional or real-time RT-PCR. ZIKV has been successfully identified with Flavivirus RT-PCR assays which target the E-encoding gene, the NS1, NS3 and the NS5 encoding gene.[91]

Nipah Virus (NiV)

NiV is an RNA virus that belongs to the *Henipavirus* genus of family *Paramyxoviridae* which causes serious respiratory diseases and deadly encephalitis in humans. The genus also includes cedar virus and Hendra virus (HeV).[92] The Henipavirus contain linear and negative-stranded RNA genome of about 18 Kb in size. The 3'-5 'RNA genome contains six consecutive gene arrangements, nucleocapsid (N), phosphoprotein (P), matrix (M), fusion glycoprotein (F), attachment glycoprotein (G), and long polymerase (L) or RNA polymerase, in order 3'-N-P-M-FG-L-5.[93] There are three predicted non-structural proteins, C, V, and W, all encoded with P gene.[94]

Diagnosis

The laboratory techniques used for detection of NiV in infected body fluids include virus isolation, immunohistochemistry, electron microscopy, serum neutralization test, viral antigen detection by ELISA, and Various real-time RT-PCR tests.[95] NiV is considered as biosafety level 4 (BSL4) agent. In 2018, Fischer *et al.* developed an indirect ELISA which was based on truncated G protein of NiV and HeV and full-length NiV nucleocapsid (N) protein.[96] A real-time PCR has been designed to identify NiV replicative viral RNA that eliminates viral mRNA amplification and targeting intergenic region splitting the viral proteins F and G.[97] An isothermal (65ºC) reverse transcription-LAMP (RT-LAMP) assay was also designed to detect all known NiV strains sequences that targeting the nucleocapsid protein gene.[98] This assay provides results within 45 min without any cross-reactivity. Immunostaining or various neutralization tests (PRNT, microtiter neutralization, or immune plaque assay) are used to characterize virus isolate and differentiate cross reactivity with other Henipaviruses.[99]

Ebola virus (EBOV)

EBOV of genus *Ebola virus* belong to *Filoviridae* family in the order *Mononegavirales*. The virus contain negative single stranded RNA genome of about 18-19Kb in size. It consist of 3' leader sequence, seven encoded proteins (Nucleoprotein, viral proteins- VP35, VP40, VP30, VP24, glycoprotein, and polymerase) and 5' tailer sequence.[100] There are five different species of EBOV: *Zaire ebolavirus Bundibugyo ebolavirus, Reston ebolavirus, Sudan ebolavirus, and Tai Forest ebolavirus and out of these only* three *strains* (Bundibugyo, Sudan, and EBOV) are majorly responsible for ebola outbreaks in human.[101] Fruit bats belonging to the family Pteropodidae are considered to be the primary hosts of EBOV.

Diagnosis

Laboratory methods which could be used to diagnose the EBOV infection include antigen-capture detection tests, antibody-capture ELISA, RT-PCR assay, serum neutralization test, virus isolation, and electron microscopy.[102] As the samples obtained from infected patients are therefore a bio-hazard risk, tests must be carried out on inactivated samples under complete biological containment conditions. Viral RNA and viral antigen detections are the primary procedures currently been considered for accurate diagnosis of EVOD. RT-PCR is regarded as the gold standard procedure for confirmation of EVD but the drawback of this method is that manual extraction and technical knowledge are necessary. The automated RT-PCR assay which got emergency use authorization (EUA) by WHO and FDA are described below:

The GeneXpert (Cepheid, Sunnyvale, CA) assay developed to detect EBOV in whole blood and oral fluids. It requires less preparation to run has 15–25°C storage specifications and test findings are accessible in <90 min.[103] The target sequence for this assay is EBOV *NP* and *GP* nucleic acids**.** The IFilmArray (BioFire Diagnostics and BioFire Defense) Biothreat-E test and FilmArray NGDS BT-E are fluorescent nested multiplex RT-PCR assay target at EBOV nucleic acid and is used to detect EBOV in, plasma, serum, whole blood, and urine.[103,104] RealStar ® Filovirus Screen RT-PCR Kit 1.0 developed by altona Diagnostics GmbH is an *in vitro* screening test used in human EDTA plasma to diagnose and distinguish Ebola and Marburgvirus-specific RNA.[105]

Human Immunodeficiency Virus (HIV)

The HIV is categorized in the *Retroviridae* family, the *Orthoretrovirinae* subfamily.[106] HIV is divided into two types; HIV 1 and HIV2. HIV consist of linear, monopartite, dimeric ssRNA(+) genome of 975 Kb with 5' cap, and 3' poly-A tail. The HIV genome also includes Long Terminal Repeats at its both ends which contain U3, R and U5 regions, 5' end for primer binding, and a polypurine tract at 3' end.[107] HIV has several major structural protein coding genes found in all retroviruses, and a number of non-structural ("accessory") genes distinctive to HIV.[108] The structural genes include gag, pol and env genes, and gag gene encodes for gag polyprotein and is processed to MA (matrix protein, P17), CA (capsid, P24), SP1 (space peptide 1, p2), NC (nucleocapsid protein, p7), and SP2 (space peptide 2, p1) and p6. The pol gene codes for viral enzymes protease, integrase, reverse transcriptase, and RNase H and env gene codes for gp160, which is processed to gp120 (surface protein) and gp41 (transmembrane protein). The non-structural proteins include accessory and regulatory proteins. Accessory proteins consist of Nef (negative factor), Vif (virion infectivity factor), Vpr (Viral protein R), Vpu (viral protein U), and regulatory proteins include Rev-ART/TRS (anti repression transactivator protein) and Tat (transactivating regulatory protein).[109]

Diagnosis

HIV infection is identified by recognizing HIV-specific serum or plasma antibodies or by observing nucleic acid using PCR or p24 antigen analysis. P24 antigen technique is achieved using a combination of monoclonal or polyclonal antibodies using the ELISA sandwich technique. These tests enable the detection of HIV p24 at a sensitivity of 10 pg/ml.[106] Some effective tests, such as neutralization or NAT, must also be performed to confirm the positive p24 test. Existing techniques to screen P24 antigen include: Enzyme-linked fluorescent assay (ELFA), ELISA, electrochemiluminescence immunoassay (ECLIA), and rapid identification of antigen and antibody.[110]

Molecular techniques comprising quantitative and qualitative HIV screening are mainly focused on NASBA, PCR, TMA, and branched chain (b) DNA approaches.[111] FDA has licensed numerous NAT assays and the most recent is NGI UltraQual® Multiplex PCR Assay for HCV, HIV-1, HIV-2, and HBV by National Genetics Institute which can screen plasma HCV, HBV, HIV-1, and HIV-2.[112] APTIMA HIV-1 RNA Qualitative Assay by Gen-Probe, Inc is an *in vitro* test for detection of nucleic acid of HIV-1 in human plasma.[113]

HIV EIA

EIA is an excellent assay used to detect anti HIV antibodies. The antibodies mainly detected are IgG subtypes.[114] In this test, the serum of the patient is incubated with an HIV antigen and, if a person is infected with the virus, the serum anti-HIV antibodies bind to the HIV antigen. The patient's antibody is subsequently detected either by an anti-human enzyme label antibody or by an enzyme labeled antigen. Genetic Systems HIV-1/HIV-2 Plus O EIA manufactured by Bio-Red laboratories is an authorized product for detection of antibodies to both types of HIV: Types 1 and 2 in human serum and plasma.[115]The disadvantage of this assay is that it may produce false results hence a confirmatory test (western blot) must be done to check for false positive results and it could followed by immunofluorescence assay, providing a confirmation to western blot**.**

Conclusion

Through this review, we have elucidated that influential prevention and disease control strategies should be introduced in the society to reduce the risk of life threatening viral infections. In view of the recent pandemic, there is an urgent need for extensive research on viral pathogens and factors influencing the disease progression so that specific vaccines and diagnostics protocols could be designed for early detection and treatment of infected individual. An increased constant vigil, against potential viral entities that could lead to large scale spread needs to be set up in the post pandemic era to curb a recurrence. Numerous advance and rapid diagnostic tools are available that have revolutionized the field of laboratory sciences. The initial high expense in setting up these Hi-Tech laboratories and equipment, as well as, trained manpower dedicated to run these facilities need a priority based cost benefit calculation and forethought keeping human well-being at paramount importance. The absence of adequate policies, and cost consideration factors alone, without weighing in the health and indirect societal benefits of having a robust health infrastructure have led to delays in the introduction of novel approaches and techniques in larger part of the developing world and in the endemic regions. There is also a need to design low cost, rapid diagnostic tests by these developing countries without compromising the quality and precision of the diagnostic assays to reduce the risk of transmissions, morbidity and mortality due to viral infections. These diagnostic tools can be utilized in epidemiological surveys to better understand the herd immunity at a given time against the infectious viral pathogen and draft appropriate health care policies. The advancement in technological capabilities should also go hand in hand with health promotion policies and awareness drives to increase knowledge about the significance of early detection and routine screening of people at high risk.

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