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## Novel Approaches are Needed to Develop the Future Methods for Diagnosis of Viral Infections

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### Abstract

Diagnostic virology has now entered the mainstream of medical practice. Multiple methods are used for the laboratory diagnosis of viral infections, including viral culture, antigen detection, nucleic acid detection, and serology. The role of culture is diminishing as new immunologic and molecular tests are developed that provide more rapid results and are able to detect a larger number of viruses. This review provides specific recommendations for the diagnostic approach to clinically important viral infections. Rapid, sensitive and rational virus detection and quantification methods are needed. Broadly targeted methods are the major theme of this review. They can reduce the time and cost of diagnosis of infectious disease. Once an accurate diagnosis has been reached, appropriate medical action can be taken.

**Keywords:** Viral infection; diagnosis; serology; Molecular diagnosis; DNA; RNA; QPCR.

### 1. Introduction

A virus is a small infectious organism that must invade a living cell to reproduce (replicate). The virus attaches to a cell, enters it, and releases its genetic material such as DNA or RNA inside the cell. The virus's DNA or RNA is the genetic material containing the information needed to replicate a new virus. The virus's genetic material takes control of the cell and forces it to replicate the virus. The infected cell usually dies because the virus keeps it from performing its normal functions. When it dies, the cell releases new viruses, which go on to infect other cells.

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Viruses are classified as DNA viruses or RNA viruses, depending on whether they use DNA or RNA to replicate. Some viruses do not kill the cells they infect but instead alter the cell's functions [1-4]. Sometimes the infected cell loses control over normal cell division and becomes cancerous [5]. As well certain viruses, such as herpesviruses and HIV leave their genetic material in the host cell, where the material remains dormant for an extended time (called latent infection). When the cell is disturbed, the virus may begin replicating again and cause disease [6-12].

Viruses usually infect one particular type of cell. For example, common cold viruses infect only cells of the upper respiratory tract. Additionally, most viruses infect only a few species of plants or animals. Some infect only people. Many viruses commonly infect infants and children. The most common viral infections are: Respiratory infections (Infections of the nose, throat, upper airways, and lungs) are more likely to cause severe symptoms in infants, older people, and people with a lung or heart disorder. Other viral respiratory infections include influenza and pneumonia [13, 14].

In small children, viruses also commonly cause inflammation of the upper and lower airways. Other viruses infect other specific parts of the body such as: Gastrointestinal tract: Infections of the gastrointestinal tract, such as gastroenteritis, are commonly caused by viruses, such as noroviruses and rotaviruses [15]. Liver: These infections result in hepatitis. Nervous system: Some viruses, such as the rabies virus and the West Nile virus, infect the brain, causing encephalitis. Others infect the layers of tissue that cover the brain and spinal cord, causing meningitis or polio [16]. Skin: Viral infections that affect only the skin sometimes result in warts or other blemishes. Many viruses that affect other parts of the body also cause a rash. Some certain viruses typically affect many body systems. Such viruses include enteroviruses (such as coxsackieviruses and echoviruses) and cytomegaloviruses [17, 18].

### ***1.1. Oncogenicity:***

Oncogenicity refers to viruses that may cause cancers. Generally, the viruses that associated with malignancies are known as tumorviruses. Some viruses alter the DNA of their host cells in a way that helps cancer develop. Only a few viruses are known to cause cancer. It is estimated that close to 20% of human cancers are due to infections with known pathogens, mainly with viruses. Currently six viruses are classified as “carcinogenic to humans” by the International Agency for Research on Cancer (IARC). These are the high-risk human papilloma viruses (HPV), associated with quasi 100% of cervix uteri tumours; hepatitis B (HBV) and C (HCV) viruses, together responsible for 80% of hepatocellular carcinoma [5, 19, 20]; Epstein-Barr virus (EBV), a causative agent of Burkitt lymphoma, some Hodgkin lymphomas, nasopharyngeal carcinoma, and some gastric cancers; Kaposi sarcoma virus (KSHV), a herpes virus associated with Kaposi sarcoma and human T-cell leukaemia/lymphoma virus type 1 (HTLV-1) that causes adult T leukaemia/lymphoma [20].

Viruses are transmitted in various routes such as fecal-oral route [21, 22], inhalation, sexually, during transfusion of contaminated blood and through vectors such as mosquitoes or ticks. Many viruses that were once present in only a few parts of the world are now spreading. These viruses include chikungunya virus, Crimean-Congo hemorrhagic fever virus, Japanese encephalitis virus, Rift Valley Fever virus, West Nile virus, Ross River virus, Zika virus, and louping ill virus.

These viruses are spreading partly because climate change has resulted in more areas where the mosquitoes that spread the viruses can live. Also, travelers may be infected, then return home and be bitten by a mosquito, which spreads the virus to other people. Chickungunya virus, which is spread by mosquitoes, was first identified in Africa but has recently spread to the Caribbean and Central, South, and North America [23, 24].

## **2. Viral diagnostic methods**

Diagnostic methods generally belong to one of four categories: Virus isolation, Virus antigen detection, Virus nucleic acid detection and Virus antibody detection (serology). Virus isolation is a rather cumbersome and slow technique. Virus antigen detection is more rapid, but still is manually intensive and relatively insensitive. Virus serology is an indirect approach with many limitations. There is a great clinical need to develop new virus diagnostic techniques.

A viral test is performed to find out the infection-causing viruses. Viruses grow only in living cells. Viruses cause disease by destroying or damaging the cells they infect, damaging the body's immune system, changing the genetic material (DNA or RNA) of the cells they infect, or causing inflammation that can damage an organ. Viruses cause many types of diseases, such as human immunodeficiency virus (HIV), cold sores, chickenpox, measles, flu (influenza), and some types of cancer.

Several types of assays may be used to investigate the presence of the viruses in each specimen such as: antibody, antigen detection, viral culture and viral genetic material (DNA or RNA). The genetic material test can show the exact virus causing an infection [25, 26].

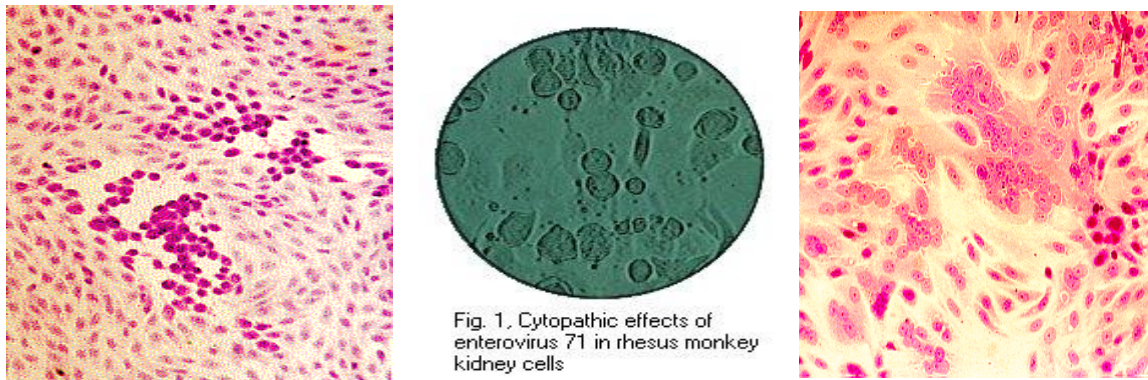
Different types of samples are used for a viral test, including blood, urine, stool (feces), organ tissue, spinal fluid, and saliva. The type of sample used for the test depends on the type of infection that may be present.

Virus Isolation : Cell cultures, eggs, and animals may be used for isolation. However eggs and animals are difficult to handle and most viral diagnostic laboratories depend on cell culture only. There are 3 types of cell cultures: (i) Primary cells - e.g. Monkey Kidney. These are essentially normal cells obtained from freshly killed adult animals. These cells can only be passaged once or twice. (ii) Semi-continuous cells - e.g. Human embryonic kidney and skin fibroblasts. These are cells taken from embryonic tissue, and may be passaged up to 50 times. (iii) Continuous cells - e.g. HeLa, Vero, Hep2, LLC-MK2, BGM. These are immortalized cells i.e. tumour cell lines and may be passaged indefinitely.

Primary cell culture is widely acknowledged as the best cell culture systems available since they support the widest range of viruses. However, they are very expensive and it is often difficult to obtain a reliable supply. Continuous cells are the most easy to handle but the range of viruses supported is often limited.

The presence of growing virus is usually detected by: (i) Cytopathic Effect (CPE) - may be specific or non-specific e.g. HSV and CMV produces a specific CPE, whereas enteroviruses do not. (ii) Haemadsorption - cells acquire the ability to stick to mammalian red blood cells. Haemadsorption is mainly used for the detection of influenza and parainfluenzaviruses.

Confirmation of the identity of the virus may be carried out using neutralization, haemadsorption- inhibition, immunofluorescence, or molecular tests.



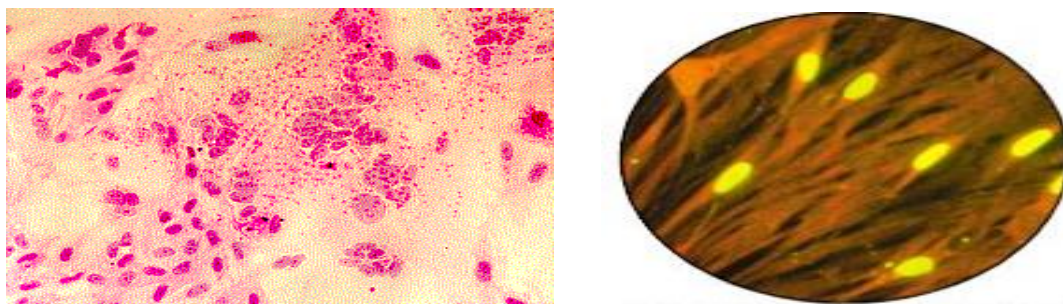
**Figure 1:** Left to Right: Cytopathic effect of HSV, enterovirus 71, and RSV in cell culture.

### 3. Limitation with cell culture

The main problem with cell culture is the long period (up to 4 weeks) required for a result to be available. Also, the sensitivity is often poor and depends on many factors, such as the condition of the specimen, and the condition of the cell sheet. Cell cultures are also very susceptible to bacterial contamination and toxic substances in the specimen. Lastly, many viruses will not grow in cell culture at all e.g. Hepatitis B and C, Diarrheal viruses, parvovirus etc.

### 4. Rapid Culture Techniques

Rapid culture techniques are available whereby viral antigens are detected 2 to 4 days after inoculation. Examples of rapid culture techniques include shell vial cultures and the CMV DEAFF test. In the CMV DEAFF test, the cell sheet is grown on individual cover slips in a plastic bottle. After inoculation, the bottle then is spun at a low speed for one hour (to speed up the adsorption of the virus) and then incubated for 2 to 4 days. The cover slip is then taken out and examined for the presence of CMV early antigens by immunofluorescence [27, 28].



**Figure 2:** Left: Haemadsorption of red blood cells onto the surface of a cell sheet infected by mumps virus. Right: Positive CMV DEAFF test. Centrifugation culture fixed and stain 16 hrs after inoculation showing viral proteins in nuclei of infected human fibroblast cells.

The role of cell culture (both conventional and rapid techniques) in the diagnosis of viral infections is being increasingly challenged by rapid diagnostic methods i.e. antigen detection and molecular methods. Therefore, the role of cell culture is expected to decline in future and is likely to be restricted to large central laboratories.

## **5. Serology**

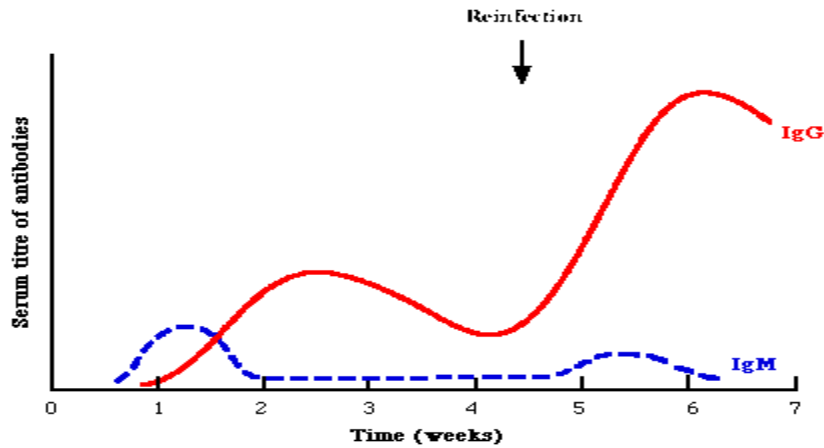
Serology forms the mainstay of viral diagnosis. This is what happens in a primary humoral immune response to antigen. Following exposure, the first antibody to appear is IgM, which is followed by a much higher titre of IgG. In cases of reinfection, the level of specific IgM either remains the same or rises slightly. But IgG shoots up rapidly and far earlier than in a primary infection. Many different types of serological tests are available. With some assays such as EIA and RIA, one can look specifically for IgM or IgG, whereas with other assays such as CFT and HAI, one can only detect total antibody, which comprises mainly IgG. Some of these tests are much more sensitive than others: EIAs and radioimmunoassays are the most sensitive tests available, whereas CFT and HAI tests are not so sensitive. Newer techniques such as EIAs offer better sensitivity, specificity and reproducibility than classical techniques such as CFT and HAI. The sensitivity and specificity of the assays depend greatly on the antigen used. Assays that use recombinant protein or synthetic peptide antigens tend to be more specific than those using whole or disrupted virus particles[24, 29-34].

### **5.1. Criteria for diagnosing Primary Infection**

- A significant rise in titre of IgG/total antibody between acute and convalescent sera - however, a significant rise is very difficult to define and depends greatly on the assay used. In the case of CFT and HAI, it is normally taken as a four-fold or greater increase in titre. The main problem is that diagnosis is usually retrospective because by the time the convalescent serum is taken, the patient had probably recovered.
- Presence of IgM - EIA, RIA, and IF may be used for the detection of IgM. This offers a rapid means of diagnosis. However, there are many problems with IgM assays, such as interference by rheumatoid factor, re-infection by the virus, and unexplained persistence of IgM years after the primary infection.
- Seroconversion - this is defined as changing from a previously antibody negative state to a positive state e.g. seroconversion against HIV following a needle-stick injury, or against rubella following contact with a known case.
- A single high titre of IgG (or total antibody) - this is a very unreliable means of serological diagnosis since the cut-off is very difficult to define.

### **5.2. Criteria for diagnosing re-infection/re-activation**

It is often very difficult to differentiate re-infection/re-activation from a primary infection. Under most circumstances, it is not important to differentiate between a primary infection and re-infection. However, it is very important under certain situations, such as rubella infection in the first trimester of pregnancy: primary infection is associated with a high risk of fetal damage whereas re-infection is not. In general, a sharp large rise in antibody titres is found in re-infection whereas IgM is usually low or absent in cases of re-infection/re-activation.



**Figure 3:** Serological events following primary infection and reinfection. Note that in reinfection, IgM may be absent or only present transiently at a low level.

### 5.3. Limitations of serological diagnosis

How useful a serological result is depends on the individual virus.

- For viruses such as rubella and hepatitis A, the onset of clinical symptoms coincide with the development of antibodies. The detection of IgM or rising titres of IgG in the serum of the patient would indicate active disease.
- However, many viruses often produce clinical disease before the appearance of antibodies such as respiratory and diarrhoeal viruses, any serological diagnosis would be retrospective and therefore will not be that useful.
- There are also viruses which produce clinical disease months or years after seroconversion e.g. HIV and rabies. In the case of these viruses, the mere presence of antibody is sufficient to make a definitive diagnosis.
- Time consuming as well extensive antigenic cross-reactivity between related viruses may lead to false positive results,



**Figure 5:** Microplate ELISA: coloured wells indicate reactivity. The darker the colour, the higher the reactivity

## **6. Virus Counter**

Virocyt (<http://www.virocyt.com>) has developed a flow cytometer-like device (Virus Counter; VC) suitable for virus quantification in liquid samples. Recently, a modified version of the device has been presented, better suited for industrial applications (for more info see <http://virocyt.com/news/new-rapid-virus-counter-system-introduced-at-world-vaccine-congress/>). VC uses two separate fluorescent dyes to stain virus samples: one specific for proteins, the other for nucleic acids. Both, a certain size of virus (>25 nm) and a certain length of viral genome (>9000 nt/bp) are necessary to guarantee a sufficient level of staining for detection. Only events positive in both channels are counted as viral particles. The non-specific staining process eliminates the need for target-specific reagents such as fluorescence-labelled antibodies required for conventional flow-cytometry methods and is therefore more cost-effective [28, 32, 35].

### **6.1. Reverse-transcription polymerase chain reaction (RT-PCR)**

The RT step is critical for sensitive and accurate RNA quantification. The amount of complementary DNA (cDNA) produced by the reverse transcriptase must accurately represent RNA input amounts. Therefore, the dynamic range, sensitivity and specificity of the RT enzyme are prime considerations for a successful RT-PCR assay. Protocols using a one tube/one enzyme-based approach are significantly more convenient than those using two tube/two enzyme based protocols but have been reported to be less sensitive [36]. RT reactions are usually carried out between 40°C and 50°C and at these low temperatures there can be problems with a somewhat lower specificity of the RT reaction. RT-PCR can be primed using specific primers, random hexamer or oligo dT primers [37, 38]. If the target RNA contains extensive secondary structure, it is advisable to use a single tube ('one-enzyme/one-tube') system utilising *Thermus thermophilus* (*Tth*) polymerase, a DNA polymerase with intrinsic RT, but no RNase H, activity) [39]. This assay uses bicine buffers containing Mn<sup>2+</sup> ions that are compatible with both RT and subsequent PCR [40]. This permits the RT reaction to be carried out at increased temperatures using primers with significantly higher *T<sub>m</sub>*, while reducing both hands-on time and the likelihood of introducing contaminants into reaction mixtures. There is also evidence that RT-PCR reactions using *Tth* polymerase may be more robust and resistant to inhibitors present in biological specimen [36, 41-44].

### **6.2. Multiplex real-time PCR**

Multiplex real-time PCR is the amplification of more than one target in a single reaction tube by using as many as four dual labeled probes in the same reaction tube. Fluorescent dyes with different emission spectra are attached to the different probes. Multiplexing reduces the handling of the samples, prevents the opportunities for laboratory contamination and the reagent costs. Multiplex TaqMan<sup>®</sup> assays can be performed using multiple dyes with distinct emission wavelengths. Available dyes for this purpose are FAM, ROX, TET, Cy3, Cy5 and JOE. TAMRA is generally reserved to be the quencher on the probe. The combination of FAM (reporter) and TAMRA (quencher) is a first choice [45, 46].

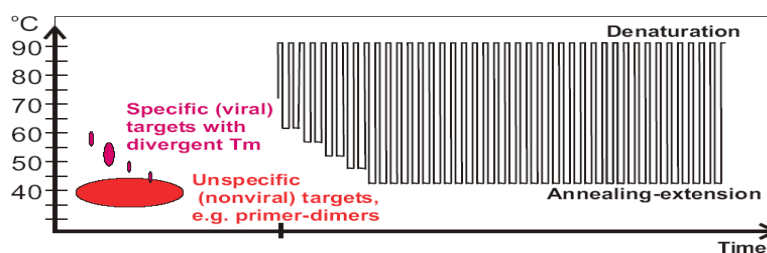
### **6.3. Touch-down quantitative PCR**

Touch-Down (TD) PCR is a method for optimizing PCR by circumventing spurious priming during amplification even if the degree of primer–template complementarity is not fully optimal.



In some systems, amplification yields were significantly increased compared to a non-touch-down procedure [20]. TD-QPCR starts with a high annealing temperature for the first primer-annealing step. It can reduce non-specific amplification and improve specificity and product yield for variable target sequence. The touch-down procedure was designed to achieve more stringent conditions in the first few cycles, to ensure efficient and specific amplification in low-copy-number but well-fitting targets in the sample. The annealing temperature is then gradually lowered to a level that increases amplification yield, and includes also less well-fitting targets [45, 47-52].

## Touch-Down PCR



The principle is to allow targets with higher  $T_m$  to amplify first, avoiding the potentially great mass of low- $T_m$  false targets, like primer-dimers.

### 6.4. The advantages of real-time PCR

Theoretically, there is a quantitative relationship between the amount of starting target sequence and the amount of PCR product at any given cycle. In practice, though, it is hard to obtain a reliable quantification with traditional (non-real-time) PCR. Real-time PCR requires less hand on and assay times. It also has a lesser risk of cross contamination since the complete assay, including the RT-step, can be performed using a single, closed, tube. Assay data are collected automatically through the closed lid. In this work, the possibility of combining contamination protection and quantification in a single PCR tube was explored. Quantification is strength of real-time PCR. Determining Ct values by following the real-time kinetics of PCR diminishes the need for a competitor to be co-amplified with the target. The use of Ct values also expands the dynamic range of quantitation up to around seven orders of magnitude, because data are collected for every cycle of PCR. Compared to endpoint quantitation methods, real-time PCR offers streamlined assay development, reproducible results, and a wider dynamic range. A sensitive system of real-time polymerase chain reaction for quantification viral RNA amount extracted from various types of human specimens was developed and established in order to lay the ground work for future studies.

There are major advantages of using QPCR compared to conventional semi-quantitative PCR. Firstly, the progression of the PCR reaction may be monitored after each cycle rather than at the end, thereby providing a much better quantification assay; secondly, very little nucleic acid is needed; thirdly it is a non-radioactive assay; and finally it can be performed in approximately two to three hours. The principle used in this assay can be adapted to a large number of biological systems and could therefore have a significant impact on patient care by providing accurate results in a shorter time. The methods developed are rapid and broadly applicable to all specimens and infected tissues.



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