Detection of RNA Viruses: Current Technologies and Future Perspectives

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ABSTRACT: RNA viruses constitute one of the major classes of pathogenic organisms causing human diseases, with varying degrees of severity. This review summarizes the conventional and emerging technologies that are available for the detection of these organisms. Cell culture-based techniques for viral detection have been popular since their inception and continue to be the gold standard against which all other techniques. Over many years, these techniques have undergone some radical changes, reducing the total time needed for detection and improving sensitivity, although even with their reliability and improved features they are being slowly replaced by nucleic acid-based technologies. These molecular detection techniques have revolutionized the area of viral detection by their high sensitivity, selectivity, and short detection time. The majority of nucleic acid-based techniques depend on amplifying viral RNA; however, there are some newer emerging techniques that detect viral RNA in live cells using various configurations of fluorescent probes. In addition, nucleic acid-based technology has made it possible for multiviral detection with either multiplex polymerase chain reaction assays or microarrays. Every technique described in this review has its own unique abilities, making them indispensable for viral detection. However, we believe that nucleic acid-based technologies will find widespread use after being standardized, limiting other technologies to very specific uses.

KEYWORDS: RNA virus, RT-PCR, NASBA, molecular beacon, cell culture, viral diagnostic

I. INTRODUCTION

Viruses are a major class of pathogenic organisms affecting human health to various degrees. The effects range from mere discomfort, leading to reduced productivity, to severe trauma and death.1,2 Some of these major viruses have RNA encased in a protein cage as their genomic material. Examples include rotavirus, influenza, measles, West Nile virus, and the human immunodeficiency virus (HIV).3 In an effort to develop effective treatment strategies and apply them to patient care, superior diagnostic methodologies are required. Early detection of the viral presence in patients provides a longer treatment window, targeted therapy, and reduces the wasteful administration of nonspecific drugs. Overall, early detection can lead to reduced treatment costs and often reduce the morbidity. Moreover, the detection and early isolation of viruses can lead to their characterization and the development of vaccine targets for preventive care.

Diagnostic techniques or RNA viruses can be broadly classified as direct, indirect, immune-detection, and fluorescence-based emerging technologies. Direct techniques involve the visual examination of viral particles in samples using electron microscopy (EM) to provide the necessary resolution. Apart from using this technique for studying viral structure or certain viral life cycle events, EM is seldom used as a diagnostic technique. Indirect methods comprise the bulk of the diagnostic technologies, and among them cell culture and nucleic acid-based amplification tests are the most widely used. Detection and isolation of viruses using cultured eukaryotic cell lines has been used since the 1960s.4 However, still remains the "gold standard." With the availability of antiviral agents, physicians now demand accurate laboratory diagnosis of their patients’ illnesses in
order to give proper treatment. Discovery of unknown viral agents still requires continued search and diligent effort. Today they still serve as the standard to which other viral diagnostic techniques are compared. These techniques are time intensive and lack the sensitivity and specificity exhibited by the nucleic acid–based molecular techniques. However their nonspecificity finds use in the isolation of new, unknown viruses/virus mixtures from suspected samples. Nucleic acid–based molecular diagnostics have revolutionized viral detection with their faster and highly sensitive diagnosis. They also provide the capability of multiplex viral detection, which can have a tremendous effect, especially in the case of viral–related respiratory tract infections. Immuno–based techniques rely on identifying host antibodies against the virus or capturing viral components using antibodies raised against them. These techniques are used widely; however, they lack the sensitivity for early detection of viral infection and are being phased out by the nucleic acid–based detection technologies.

Several emerging technologies using fluorescence–based detection are available for viruses that are hard to detect using conventional diagnostic methodologies. These fluorescence techniques are sensitive and provide the capability of viral detection in real time.

Any viral diagnostic technology is evaluated against 2 key qualities: sensitivity and selectivity. Sensitivity implies the limit of detection of the assay. Higher sensitivity helps in early detection of the virus and means it can provide conclusive diagnosis in the presence of interfering components of the sample. Depending on the nature of the detection technique, sensitivity is expressed in various measures. Selectivity is the measure of accurate determination of a particular virus/viral class in the presence of other target organisms. Sensitivity and selectivity are key attributes of molecular diagnostics/nucleic acid–based amplification tests because they can specifically detect the genomic RNA of a specific virus even in the presence of contaminating nucleic acids of other viruses or the host.

In this review we have captured the essence of viral RNA detection methodologies that are used widely and some that express huge future potential. We start with the culture–based methodologies, which still are indispensable, even with the huge influx of newer technologies. Discussion of the highly efficient nucleic acid–based technologies and emerging methodologies follows, and we end with our perspectives on future technologies.

II. CELL CULTURE

Use of cultured eukaryotic cells in a controlled environment for virus propagation and isolation is widely accepted and is still considered the gold standard. Most of the emerging techniques for viral detection are validated by comparison against the cell culture technique. The availability of a variety of cell lines for culture and their susceptibility to viral infection, antibiotics, and chemically defined cell culture media led to increased use of this technique for viral detection and isolation. Traditional and current methodology involves culturing the cells—for example, buffalo green monkey kidney cells, Madin–Darby canine kidney epithelial cells, and human lung fibroblasts (MRC–5)—as a monolayer in a variety of culture vessels. The viral infected samples of the inoculum are incubated with the monolayer for 60–90 minutes, after which the inoculum is replaced with fresh media. Depending on the type of virus, the cells are incubated for 4 to 10 days and monitored regularly for cytopathic effects (CPEs) using a microscope. Observing CPEs in unstained cell culture and relating them to a specific type of virus is still the popular way to detect viral presence and proliferation. However, for viruses that proliferate slowly and that produce very little to no CPE, additional reagents are applied to the cell monolayer to detect the infection. For viruses expressing hemagglutinating proteins like influenza and parainfluenza, the culture media is replaced with erythrocytes, which agglutinate at the sites of viral release. These hemadsorbing foci provide the indication of viral presence in as soon as 12 hours. To further classify the specific type of virus infecting the cultures displaying CPEs/hemadsorbing foci, fluorescently labeled antibodies generated against known viral antigens are
used. This process is fast and relatively simple to perform, although the main shortcoming is that it cannot differentiate serotypes of same viral strains (e.g., coxsackieviruses). In some cases, antibodies that have high binding efficiency and specificity are yet to be identified. The lack of specificity and long incubation times has taken away a considerable amount of focus on culture techniques as a diagnostic technology. However, cell culture remains the only technique available to isolate a wide variety of viruses without any prior information about their kind.

Significant effort has been applied to reduce the time frame of cell culture techniques for viral detection. Improvements have been implemented on both the culture formats and detection before observing CPEs. The shell vial is one of the most widely accepted new culture formats. It consists of a small vial with a coverslip at the bottom, on which the cell monolayer is grown. These culture formats have led to rapid detection of viruses based on enhanced viral adsorption by centrifuging the culture vial with sample at gentle speeds and using fluorescently labeled antibodies against specific viral antigens before CPE detection. In fact, this technique has been applied for the detection of cytomegalovirus, and the results are reported as early as 16 to 24 hours with 90% accuracy.

To detect multiviral infections or suspected unknown viruses, cocultured cells have been used for a variety of viruses. They are available in the shell vial format, and detection of known viral classes is mostly carried out using fluorescent antibodies. Another technique that has been developed to reduce the time to detection and increase the specificity is the use of transgenic cell lines in culture. Transgenic technology, together with increasing knowledge of the molecular pathways of virus replication, offers the possibility of using genetically modified cell lines to improve virus growth in cell culture and to facilitate detection of virus-infected cells. Genetically modifying cells so that they express a reporter gene only after infection with a specific virus can allow the detection of infectious virus by rapid and simple enzyme assays such as beta-galactosidase assays without the need for antibodies. Although transgenic cells have recently been successfully used for herpes simplex virus detection, much more work needs to be done to adapt this technology to other human viral pathogens such as cytomegalovirus and respiratory viruses. The principle involves the incorporation of a genetic element into the host cell line that is activated only by a specific viral replication event. It has been applied successfully for several viruses, including herpes simplex virus, and the results have been reported within 16 to 24 hours.

The development of nonculture techniques is being pursued rapidly, but cell culture isolation of viruses still remains a reliable standard for comparison of performance and sensitivity. In the case of culture-based detection, the sensitivity is expressed in terms of plaque-forming units (PFUs) per unit volume, wherein a plaque is an isolated zone of cell death due to viral infection. This is a more qualitative measure and does not indicate the exact number of viral particles. Also, the number of viral particles that can yield a plaque depends on their infectivity and is not the same among different viral classes. For example, for a certain virus, 1 PFU may be 50 particles, whereas it is 100 for other viruses. The newer technologies express sensitivity in terms of the number of RNA copies that can be detected and equate it to the number of particles in a PFU for comparison. Cell culture also remains the only technique available to detect unknown viruses and isolate them for further characterization.

III. NUCLEIC ACID–BASED AMPLIFICATION TESTS

Molecular techniques involving the amplification of viral genomic material have improved their detection significantly. These diagnostic methodologies are extremely sensitive and provide rapid diagnosis and multiplexing options for the detection of several viruses at same time. A very important nucleic acid–based amplification test (NAT), because of all the benefits mentioned above, can lead to targeted treatment for the patient, thus reducing the costs and improving the chances of recovery and survival by several fold. One other advantage
of these techniques, often not stressed enough, is the lack of a need to culture harmful viruses and maintain the expensive infrastructure required to do so. Tremendous progress has been made in the detection of HIV, hepatitis C virus (HCV), Human papilloma virus (HPV), hepatitis B virus (HBV), hepatitis c virus (HCV) and hepatitis E virus (HEV). In fact, in the case of viral encephalitis, it is recommended that the herpes simplex virus RNA from cerebrospinal fluid be detected using a NAT. These have been slowly replacing traditional methods in diagnostic laboratories. Upon development and implementation of suitable controls, they have the required characteristics to replace the conventional techniques completely. There are currently 2 forms of NATs that are being used and improved upon: nucleic acid sequence-based amplification (NASBA) and real-time polymerase chain reaction (PCR).

NASBA is an isothermal amplification reaction (41°C) resulting in an RNA product and uses 3 enzymes: avian myeloblastosis virus reverse transcriptase (AMV-RT), RNase-H, and T7-RNA polymerase. As illustrated in Fig. 1A, the first step involves binding a complementary primer to viral RNA, and it contains a T7 polymerase promoter region to be used in later steps. This primer (P1) is extended to form an antisense complementary DNA by the action of AMV-RT. RNase-H in the reaction hydrolyses the RNA in complex with DNA and a primer (P2) binds to the released DNA strand. This is further extended by AMV-RT, resulting in double-stranded DNA molecules that are further used as templates to make more antisense viral RNA copies by the action of T7 polymerase. The amplified product can either be detected at the end of the assay or in real time. In the former case, the amplified product can be resolved on an agarose gel and stained with ethidium bromide. For a more sensitive detection, P1 containing a region complementary to an electrochemical (ECL) detector probe can be used. Once the amplified product is obtained, an ECL probe labeled with ruthenium is added and the product is detected in a suitable ECL counter. This format of NASBA has been used for sensitive detection of West Nile virus, St. Louis encephalitis and Dengue virus. To detect the product in real time, molecular beacon (MB) probes have been employed. MBs are DNA molecules containing a target-specific region and self-complementary region to form a stem-loop structure. One end of the stem is attached to a fluorescent moiety and the other end to a quencher. In the absence of a target, the MB retains it stem-loop confirmation and the fluorescence is quenched because of its close proximity with the quencher. The target-specific region of the MB can hybridize with any single-stranded nucleotide sequence with extreme specificity: up to 1 base pair discrimination. Upon target binding, a structural change is forced, causing the fluorophore to be pulled apart from the quencher and resulting in an observable fluorescence signal.

Real-time PCR implies the amplification of complementary DNA (cDNA) obtained from viral RNA in real time. The reaction mixture contains the reverse transcriptase along with the polymerase, primers for cDNA amplification, and necessary reagents. The primers used can be specific to the viral class/strain or random hexamers. The process, as illustrated in Fig. 1B, involves the formation of double-stranded cDNA by the action of the reverse transcriptase enzyme followed by its amplification by a PCR. Several efficient reverse transcriptase and polymerase enzymes are currently available that are more robust and display high fidelity. The two most commonly used reagents for detection of amplification products include SYBR green dye and the TaqMan probe. The SYBR green dye binds to double-stranded DNA molecules being formed in the reaction and shows enhanced fluorescence that can be recorded and is directly proportional to the product formed. The use of the TaqMan probe provides an opportunity for virus-specific amplification and multiplex identification of different viruses. The TaqMan probe is a small DNA molecule with a fluorescent dye attached to one end and quencher on the other. The sequence of the probe is complementary to the cDNA originating from viral RNA and binds to it during the primer annealing. The polymerase extension of the primer leads to the displacement of the Taq-
FIGURE 1. A: Nucleic acid sequence-based amplification (NASBA). B: Real-time polymerase chain reaction molecular assay for viral detection. cDNA, complementary DNA; RT, reverse transcriptase.
Man probe and hydrolysis, leading to the release of the fluorescent dye, whose fluorescence can be recorded and is proportional to the amplification. Because of sequence specificity, the availability of a variety of fluorescent dyes, and machines capable of recording multiple fluorescent wavelengths, a mixture of different viruses can be detected in the same reaction.

Several improvements have been made to the NATs mentioned here to increase the sensitivity, reduce the assay variations, and make it a more standard technique for universal adoption. One of the most common improvements is the use of multiple primers targeting different regions of the viral RNA and internal amplification controls to account for the reverse transcriptase enzyme efficiency. The other variation involves carrying out a nested PCR rather than conventional PCR for amplification. In nested PCR, an initial amplification of cDNA is carried out using universal primers, after which the amplified sample is subjected to another PCR with primers more specific to the viral strain. This process has been shown to increase the sensitivity by 1000-fold and is commonly employed for the detection of various classes of viruses. A rather impressive feat achieved by the RT-nested PCR technique is the multiplex detection of respiratory viruses. This assay could detect 21 different viruses, including major influenza strains, in about 30 minutes and with 10- to 100-fold higher sensitivity than conventional RT-PCR. Improvements have been incorporated to reduce the time to detection. A technique called high-speed droplet PCR has been shown to detect human influenza virus in <30 minutes. The droplet PCR machine consists of 2 heating blocks with the reaction tube passing through them. The heating blocks are maintained at the different temperatures required for the PCR reaction. The reaction mixture (1 µl) is added into the reaction tube, which contains silicon oil, and thus forms a droplet that settles at the bottom. For different steps, the setup is rotated so that the droplet lies with the heating block at the right temperature. The machine is provided with a fluorescence detector that can monitor fluorescence changes at each extension step. This set up alleviates the time required for temperature increase or decrease during PCR and thus reduces the reaction time, from 80 to 28 minutes. In addition, this technique retained the sensitivity even with minimally pretreated patient samples, thus reducing the overall detection time.

The NAT assays have proved to be sensitive, rapid, and cost-effective diagnostic techniques. However, there are still a number of technical challenges to adapt these techniques universally. First, this technique cannot be used to identify a new virus strain, as opposed to the culture technique. Second, viral RNA-based detection remains a challenge for viruses that show huge genetic variability, like HIV. Some conserved regions of the HIV genome have been proposed for primer design, but that does not cover all the HIV-1 strains relevant to a geographic location. Carrying out the amplification reaction by reducing the amplification temperature over subsequent cycles to make the primers overlook a mismatch has been proposed as well. Last, a major limitation lies in the inefficient reverse transcription step of forming the cDNA and thus accurate estimation of viral RNA. The inconsistency of the results from RT-PCR methodology are largely due to this step, the efficiency of which is said to be about 20%. The use of proper internal control samples and the more robust reverse transcriptase enzyme will reduce the variability and make the process more universally adaptable.

IV. EMERGING TECHNOLOGIES

Microarray is a relatively new technique that has shown to be valuable in identifying and diagnosing viruses. DNA microarrays are used for the identification or quantification of specific DNA sequences in complex nucleic acid samples. DNA microarrays allow for a way to target the genomic makeup of viruses. The design may vary depending on the application, but the basic method is to isolate the RNA from a cell sample, then use reverse transcriptase PCR and fluorescently label the nucleic acid product. The resulting fluorescently labeled nucleic acids then are used to screen an array of
immobilized oligonucleotides that have been designed specifically for the genetic makeup of the virus of interest. Microarrays have successfully detected and genotyped rotaviruses, influenza, zoonotic viruses, and structured viruses (antigenically Norwalk virus [NV]-like or SMA-like human papillomavirus, and a broad spectrum of other viruses in a single assay.

High-resolution detection of nucleic acids inside the cells can allow for discriminating detection of the cells infected by the virus. One technique to achieve this sort of viral detection is accomplished by fluorescence in situ hybridization (FISH). FISH has been shown to be a reliable alternative to the traditional viral detection assays and may lack some of the concerning issues inherent in the traditional methods, such as CPE deficiencies, internal viral processing, and real-time monitoring. FISH has proven the ability to detect and localize specific viral nucleic acids within cells. The general FISH protocol calls for a fixation agent, usually pentaformaldehyde, to fix the cell and its internal components, allowing for the localized detection of viral RNA or messenger RNA inside cells upon the membrane becoming permeable. This technology can be particularly beneficial when studying the life cycles of viruses because it allows the locations of nucleic acids at certain time points during the infection to be pinpointed. The FISH method has been used for the detection of virus-specific nucleic acids, using fluorescently labeled oligonucleotides, for a wide variety of viruses including Epstein-Barr, Dengue, HIV, and the poliovirus.

To achieve higher specificity and sensitivity over traditional FISH methods, variations have been performed, for instance the use of locked nucleic acids (LNAs) in combination with flow cytometric fluorescence visualization. LNAs are RNA molecule analogs that contain a methylene bridge that connects the 2'-oxygen of ribose with a 4'-carbon. This bridge results in a locked 3'endoconformation, which reduces the conformation flexibility of the ribose and increases the degree of local organization of the phosphate backbone. This entropic constraint ultimately results in increased sensitivity and specificity with a complementary target. The synthesis of LNA allows for LNA bases to be interspersed among DNA and RNA and for the susceptibility to RNase-H to be optimized for individual applications. A single LNA substitution has resulted in an increase in the melting temperature by as much as 9.6°C. The use of LNA-based probes for RNA detection provides the distinct advantages of higher binding affinity and increased stability as opposed to traditional nucleic acid probes. Using fluorescently labeled LNAs along with in situ monitoring via flow cytometry, the viral RNA expression of the Sindbis virus was detected accurately over time.

Other techniques similar to FISH have proven to be particularly beneficial when studying the life cycles of viruses. In particular, the use of MBs for viral detection in live cells allows for real-time monitoring. The MBs used in live cell applications are the same as those previously described, except with particular design modifications to allow for intracellular delivery and nuclease resistance. The modifications to intracellular use may include the addition of a cell-penetrating peptide, for example, tat peptide, through a thiol-maleimide linkage, as shown in Fig. 2A. The nuclease resistance properties come by altering the phosphodiester bond by replacing the nonbridging oxygen with sulfur along with the substitution of the 2'-sugar deoxy with a 2'-O-methyl group (Fig. 2A). The tat-modified, nuclease-resistant MBs described were used to detect the real-time infection of coxsakievirus B6 by targeting the viral RNA in live buffalo green monkey kidney cells (Fig. 2B). MBs also have shown the ability to detect viral replication in a wide variety of other viruses by targeting virusespecific messenger RNA in live cells. A TAT peptide-delivered molecular beacon was developed and utilized to enumerate murine norovirus 1, a human norovirus (NoV).

Other recent alternatives to FISH methods also are capable of highly sensitive viral detection. Some of these methods include RNA oligonucleotide nanoparticles and 2-color nanoparticle probes. RNA oligonucleotide nanoparticles have been used to detect the (+) single-stranded
RNA of HCV. This method works through the immobilization of the HCV RNA-dependent RNA polymerase (NS5B) onto a glass chip via protein terminal modification. Subsequently, quantum dot-supported RNA oligonucleotides are flown over the immobilized NS5B, allowing for NS5B-target RNA binding signaling. The detection limit of the system has been determined to be around 1 ng mL⁻¹.⁶ The 2-color nanoparticle probes have been used for rapid and sensitive detection of respiratory syncytial virus particles.⁶⁸ The system uses 2 fluorescent nanoparticles capable of excitation at the same wavelength and emission at different wavelengths. For detection of respiratory syncytial virus (RSV), the particles were coupled with anti-RSV F or anti-RSV G monoclonal antibodies, which were allowed to interact with RSV then flown through a microcapillary tube; the confocal probe volumes were identified by laser excitation. This method works based on the concept that if 2
nanoparticles are moving freely in solution, the photons they emit will arrive at the detector at different times, whereas nanoparticles bound to the same target will arrive at the target at the same time, thus, allowing for low-concentration detection of virus particles by time-coincident photons. This system also was able to compare the relative level of surface F and G proteins for RSV mutants, which poses the potential for a multiplexed system capable of detecting and differentiating various viruses.68

Although we have discussed methods to detect and visualize viral nucleic acid synthesis and processing through the cell, there are still some limitations to the sensitivity of most of the probes presented. To visualize single copies of nucleotide molecules, very particular equipment often is required, such as the prism-type total internal reflection microscope.69 A recent study reported the ability to determine the number of viral RNA copies packaged into a single influenza particle using a multicolored FISH probe.70 The determination of the RNA copy in a single viral particle was achieved using total internal reflection microscopy by measuring the photobleaching steps of the individually fluorescent viral particles containing the hybridized viral RNA segments. Another recent study reported the detection of single viral RNA molecules of influenza as early as 20 minutes after infection using only a standard fluorescence microscope based on a novel design in conjunction with FISH.71 The method uses a Polymeric Sequence Probe (PSP) capable of amplifying the signal of a single viral RNA to the point that it can be visualized with a typical fluorescence microscope. The polymeric probe is constructed using rolling circle amplification to produce thousands of repeating target nucleotide sequences specific to the influenza viral RNA and tandem fluorophore-tagged, single-stranded DNA, as shown in Fig. 3A. This design essentially clusters the thousands of fluorescent molecules per polymeric probe into one single sharp signal. The experiment was performed by fixing the infected cells with paraformaldehyde for the direct immobilization of the viral RNA inside the cell. Cell fixation and permeabilization at different time points after infection allowed the viral RNA expression level and the cellular locations of single viral RNA molecules of influenza A virus in Madin-Darby canine kidney epithelial cells to be monitored (Fig. 3B).71

V. FUTURE PERSPECTIVES

There are a number of ways to detect RNA viruses, and each has certain benefits and disadvantages. Traditional EM methods are used solely to characterize viral structure and life cycle. The equipment and maintenance required can be very expensive and the sensitivity is often quite low for them to be considered viable detection methodologies. Since its inception, cell culture has remained an important and indispensable tool for RNA viral detection. There has been tremendous effort to reduce the detection time and increase the sensitivity of the cell culture–based assay. The use of fluorescently labeled antibodies and shell vials have led to virus identification before CPEs. Even with all the breakthroughs the cell culture techniques have undergone, the introduction of nucleic acid–based viral detection techniques have taken away dependence on them. These molecular detection methods are several fold more sensitive and selective than the cell culture–based methods. These techniques also have been refined and the time of detection has been brought down to as little at 30 minutes. They have been adapted to replace cell culture and become the test of choice for the detection of certain viral classes, and this number will continue to rise in future. However, the current viral diagnostic field cannot completely replace cell culture techniques because they are still considered the gold standard to which all other techniques are compared. At present, cell culture techniques are the only reliable method for isolating unknown and emerging viral strains. NATs have a huge disadvantage in that majority of the reported assays do not have reliable controls and can vary from laboratory to laboratory. There is a need for universally accepted standards and quality control of the reagents used. We believe that until reliable standards and quality control procedures are established for
nucleic acid–based techniques, they have a long way to go before they can be adopted into routine use in diagnostic laboratories. Meanwhile, the cell culture–based techniques should be retained and used as a final confirmatory mechanism. Once the molecular diagnostics have been standardized, cell culture facilities can be centralized for just viral isolation and detection of new strains. Microarrays are another method that has been adopted widely for determining virus infection and genotype. Microarrays can offer a sensitive and relatively quick method for detecting viruses, but since they rely on cell lysis, they cannot offer information about the viral viability and the relationship between the cell and cytopathic phenotypes. The emerging methods that yield the most insight on real-time virus propagation and have the capability to detect single viral RNA molecules involve the use of fluorescent
probes and live or fixed cells. Although some of these new methods may not be ideal for diagnostic purposes, the emerging technologies are able to generate the most information for understanding of the viral propagation cycle inside cells. This elucidation of the life cycle can pave way for designing better and more reliable diagnostic technologies and treatment strategies.

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