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Review

Real-time molecular methods to detect infectious viruses

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ABSTRACT

Waterborne transmitted viruses pose a public health threat due to their stability in aquatic environment and the easy transmission with high morbidity rates at low infectious doses. Two major challenge of virus analysis include a lack of adequate information in infectivity and the inability to cultivate certain epidemiologically important viruses *in vitro*. The use of fluorescent probes in conjunction with fluorescence microscopy allows us to reveal dynamic interactions of the viruses with different cellular structures in living cells that are impossible to detect by immunological or PCR-based experiments. Real-time viral detection *in vivo* provides sufficient information regarding multiple steps in infection process at molecular level, which will be valuable for the prevention and control of viral infection.

Contents

1. Introduction

Environmental virology initiated with scientists attempting to detect poliovirus more than half a century ago [1]. In the United States, waterborne disease outbreaks were associated with treatment deficiencies in water supply and distribution system contamination [2]. Close to 50% of all waterborne disease outbreaks are due to acute gastrointestinal illness (AGI) caused by agents of undetermined etiology [3]. Given the specimen collection limitations and disease patterns, it is reasonable to speculate that most of the unknown agents may be of viral origin. Among the identified etiologic agents, the presence of human enteric viruses in water such as enteroviruses, astroviruses, hepatoviruses, rotaviruses, Norwalk and related caliciviruses, have accounted for more than half of the outbreaks and worldwide epidemics [2,4–7].

According to US centers for disease control and prevention, human enteric viruses are mainly transmitted by the fecal-oral route, such as through ingestion of contaminated food or water. Poliovirus is the causative agent of poliomyelitis (often called polio or infantile paralysis). The non-polio enteroviruses (e.g. coxsackie A/B viruses, echoviruses) cause a variety of clinical syndromes, including gastroenteritis, viral meningitis, myocarditis, encephalitis, and diabetes mellitus. Hepatoviruses cause acute liver infection. Four of the human enteric virus, coxsackievirus, echovirus, calicivirus, and adenovirus, have been included among the microorganisms of concern on the Environmental Protection Agency's (EPA) Drinking Water Contaminant Candidate List (CCL) [8]. The importance of water as a vehicle for virus transmission, coupled with low infectious doses prompt the urgent need for rapid and reliable methods to detect small numbers of infectious virus particles in environmental samples.

Abbreviations: AGI, acute gastrointestinal illness; CPE, cytopathic effects; DABCYL, 4-((4-(dimethylamino)phenyl)azo)benzoic acid; ELISA, enzyme-linked immunosorbent assay; FISH, fluorescence *in situ* hybridization; FRET, fluorescence resonance energy transfer; FMDV, foot and mouth disease virus; HIV-1, human immunodeficiency virus type 1; MBs, molecular beacons; NIR, near-infrared; pMHC, peptide-major histocompatibility complex; PL, photoluminescence; PFU, plaque forming unit; PCR, polymerase chain reaction; Qdot, quantum dot; RT-PCR, reverse transcription-PCR.

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Conventionally, immunological, nucleic acid-based, and infectivity-based (cell culture) methods, have been applied as molecular techniques for virus analysis [1,7,9–13]. Immunological and nucleic acid-based methods determine only the total virus particle number and do not stress the discrepancy between the presence of physical virus particles (irrespective of its ability to infect cells and reproduce) and viable virus [1,7]. The only reliable method to detect infectious viruses is based on mammalian cell culture, which detects the production of visible cytopathic effects (CPE). This method is difficult to perform and may take weeks before the viruses reach measurable amounts to allow detection. Epidemiologically important viruses that cannot be grow in cell culture or grown with difficulty, e.g. adenovirus type 40 and 41, astrovirus, and caliciviruses, have prompted the need for new detection approaches that are rapid, sensitive and specific. These approaches must be quantitative and can preclude the detection of non-infectious viruses.

In this review, we provide a survey of current molecular methods for near real-time or real-time detection and quantification of infectious viruses. This article does not contain details about the basic steps of sampling, concentration or the recovery of viruses from environmental samples, but rather highlights the key issues pertaining to overcoming the main difficulties for infectious viral detection and characterization such as viral diversity, occurrence of low particle numbers (particularly in the water environment), and the technical challenges of virus assays.

2. Current methods of viral detection

Scientists have been making progress in viral detection methods over the past 60 years. The advent of molecular biology further leads to the development of new approaches for meeting current challenges and has expanded our knowledge of viral structures and functions at the molecular level. A variety of experimental techniques, e.g. immuno-affinity, nucleic acid-based or cell culture-based detection, have already been employed to measure the presence of virus or viral infection. Immunological (serological) methods such as radioimmunoassay, immunofluorescence, immune electron microscopy or enzyme-linked immunosorbent assay (ELISA) are based on the interaction between a viral antigen and an antibody; the capture antibody directs against the viral antigen and the bound complex are detected via chromogenic or fluorogenic molecules. The detection limit varies by the variability of the viral genome and the affinity of antibody interaction. Immunological methods require sophisticated apparatus and specialized training, and they generally lack the degree of sensitivity required to detect the low quantities of viruses expected in environmental samples [1,7].

Substantial improvements in sensitivity over conventional molecular techniques have been achieved by nucleic acid-based amplification methods such as polymerase chain reaction (PCR), reverse transcription-PCR (RT-PCR), or quantitative real-time PCR (qRT-PCR) [9–12]. The employment of PCR-based methods for viral detection and quantification provides the benefit of rapid analysis with high sensitivity and reproducibility at relatively low cost. However, the major obstacles include: (i) environmental inhibitors (e.g. humic compounds) concentrated along with viruses during water sample processing, (ii) the small volume assayed may lead to false-negative results because of the low virus titers; and (iii) PCR or RT-PCR may yield false-positive results by detecting non-infectious or inactivated viruses, suggesting that a positive result may not necessarily pose a public health threat.

PCR amplification can be combined with other molecular technologies, e.g. *in situ* hybridization (ISH) [14,15], microarray [16], or cell culture, to maximize sensitivity and specificity in the detection of known waterborne pathogenic viruses. For example, ISH can localize and determine the relative abundance of specific DNA or RNA sequences in infected cells that are fixed on a glass slide. Fluorescence *in situ* hybridization (FISH) can be used in viral diagnostics to assess chromosomal integrity and to help the identification of viruses. To detect the low viral copy sequences, the assay sensitivity may be improved by *in situ* RT-PCR or PCR [14,15,17,18]. Studies have shown that *in situ* RT-PCR (*in situ* PCR) allows for the detection of RNA sequences of infectious bursal disease virus and human papillomavirus DNA with copy numbers below the detection threshold of conventional ISH analysis [19,20].

DNA microarray has become an alternate hybridization method for the analysis of cellular gene expression in response to viral infection. In general, microarrays are miniaturized arrays of locations on a solid surface such as a glass microscope slide or a silicon chip in aligned rows. The DNA sequences attached to a microarray are used as probes to capture their corresponding fluorophore-labeled DNA targets. Probe-target hybridization can be quantified by fluorescence-based detection to determine the relative abundance of the targets. Recently, a foot and mouth disease virus (FMDV) microarray was described to simultaneously detect seven FMDV serotypes. The results encourage the development of new oligonucleotide microarrays to probe the differences in the genetic and antigenic composition of FMDV, and to gain insight into the molecular epidemiology of this pathogen [21]. Using the fully sequenced viral genomic data, a highly conserved oligonucleotide DNA microarray is capable of simultaneously detecting and identifying diverse viruses by the unique pattern of hybridization generated by each virus. Perhaps equally important to the detection of viral pathogens, the viral genomic and microarray-based strategy has the potential to facilitate the determination of viral subtypes and to identify diseases of unknown etiology [16,22]. A subtyping assay for both the hemagglutinin and neuraminidase surface antigens of the avian influenza viruses has been developed using padlock probes to form circular molecules when paired to the appropriate target [22]. The circular probes are amplified by a rolling-circle amplification and PCR, and when combined with a microarray output for detection this assay is capable, of differentiating among all known surface antigen subtypes within 4 h. Viral microarray design can further use the Protein Families database, protein-motif (subjected to coding sequences) and nucleic acid-motif (subjected to non-coding sequences) finding algorithms to ensure a nearly complete coverage of the related viral sequence database [23].

The major drawback to most current methods is that they are usually used to approximate the quantity of viruses present in a sample but do not provide information whether a pathogen has the ability to establish an infection or not. To overcome this problem, the infectious assays may be achieved by cell culture techniques with the appropriate cell line in conjunction with other developed methods for direct assessment of infectious virus. For example, cell culture followed by RT-PCR probe the specific viral mRNA present in the cell during viral replication. Propagation of cultivable virus in host cells generates enough progeny viruses to enable ready detection by the nucleic acid-based test [13]. However, this method requires additional mRNA extraction, RT-PCR reactions, and gel analysis, leading to added analysis time and the potential for contamination. Cell culture method remains the gold standard for virus diagnosis because it is the only method available for detecting infectious viral particles and can achieve a detection limit of 1 plaque forming unit (PFU) per volume [7]. However, some health-significant viruses such as astrovirus or norovirus still cannot be cultivated or grow poorly in cell culture [17,24]. Certain viruses like hepatovirus and adenovirus have been reported that the viral replication is relatively slow and causes ambiguous CPEs in cell culture [25,26]. New cell lines need to be investigated

for those non-culturable but epidemiologically important viruses. The study of norovirus, a major cause for foodborne gastroenteritis outbreaks, has been complicated by recombination between strains and the lack of an *in vitro* culture system with high yield. Recently, a complicated norovirus cell culture model has been reported for an infectivity assay that infects and replicates in a 3D human small intestinal epithelium [17]. This breakthrough may provide insights into the molecular biology of norovirus, such as viral attachment and intracellular replication, in addition to the genomic and proteomic profiling. Alternative steps that depend on functional components of the virus needed for infection may be employed as an additional approach to detect only infectious viruses. Methods include the specific capture of virus by cellular receptors for virus *in vitro* followed by molecular detection of viral nucleic acid in the captured virus [27].

3. Emerging tools for real-time monitoring of viral replication

Real-time detection of the viral load in living cells provides information on the dynamics of proliferation of the infectious pathogen and has prognostic relevance in a number of clinical studies that can serve as a basis for guiding therapeutic interventions. In particular, the ability to monitor the real-time replication of viruses in living cells are vital for the rapid detection of viral infection and understanding of viral pathogenesis. Among the technologies currently under development for gene detection in living cells, the most promising one is perhaps molecular beacons (MBs). MBs provide a label-based and separation-free detection scheme and the specificity and sensitivity of MBs have led to their use in numerous *in vitro* hybridization assays [28–31]. They are single-stranded oligonucleotide probes possessing a stem-loop structure and are double labeled with a fluorophore at one arm and a quencher at the other. These probes are specific for a target nucleotide sequence and produce fluorescence upon target binding. The spontaneous hybridization between MBs and their target sequences is highly specific and can even distinguish a single nucleotide mismatch [32–34]. The reported MB-based reverse-transcription-PCR (RT-PCR) provided sensitive and specific detection of hepatitis A virus and as few as 1 PFU was detected [35]. Recently, MBs have been used to detect the presence of viral RNAs in infected cells with positive responses to even one single infectious viral particle (Fig. 1) [36,37]. By labeling endogenous RNA with MBs, the dynamic behavior of poliovirus (+) strand RNA in living host cells have also been studied [38].

Although MBs have the potential to become a powerful real-time tool to monitor and quantify the level of infectious virus in living cells, the major challenge in using conventional MBs *in vivo* is the relative short half-life (~50 min) of MBs due to cytoplasmic degradation. This could dramatically decrease the MBs' sensitivity by digesting the deoxyribonucleotide backbone and disrupting the stem-loop structure, resulting in false-positive fluorescence signals unrelated to MB/target hybridization [39,40]. Moreover, upon target binding, the RNA–DNA duplex region is susceptible to cellular RNase H activity; the RNase H cleavage results in false-negative signals due to the degradation of the bound RNA [41]. To maintain the stability of MB structure, many attempts, such as 2′-O-methyl modifications and phosphorothioate internucleotide linkages, can be made to increase duplex stability and nuclease resistance, as well as to have a higher affinity and coupling efficiency [42–45]. The rationale for using nuclease-resistant MBs to detect viral RNAs in living cells is to improve signal-to-noise ratios by eliminating false-positive and false-negative fluorescence signals derived from endogenous nuclease degradation.

In addition to the short half-life, real-time monitoring of viral replication is hampered by the lack of an efficient and non-invasive method for intracellular delivery of fluorescent probes. The *in situ* hybridization with MBs requires permeabilization for MB molecules to enter the cell's interior and cell fixation prior to microscopy observations; the pre-treatments make the *in vivo* localization of mRNA/RNA or real-time detection of viral replication impossible. Endocytic approaches such as transfection are slow and the probes are predominately trapped inside endosomes and lysosomes [46]. Even microinjection is not suitable for viral detection because it is difficult to predict which cells are infected "*a priori*". Cellular uptake based on streptolysin O is faster (~2 h) but can only be used in *ex vivo* cellular assays uptake, and rapid nuclear localization was observed [47]. Recently, the peptide-based delivery systems of protein transduction domains and cell penetrating peptides, such as human immunodeficiency virus type 1 (HIV-1) TAT-derived protein, have been described [48,49]. It is believed that cell-penetrating TAT peptides exhibit "non-classical import

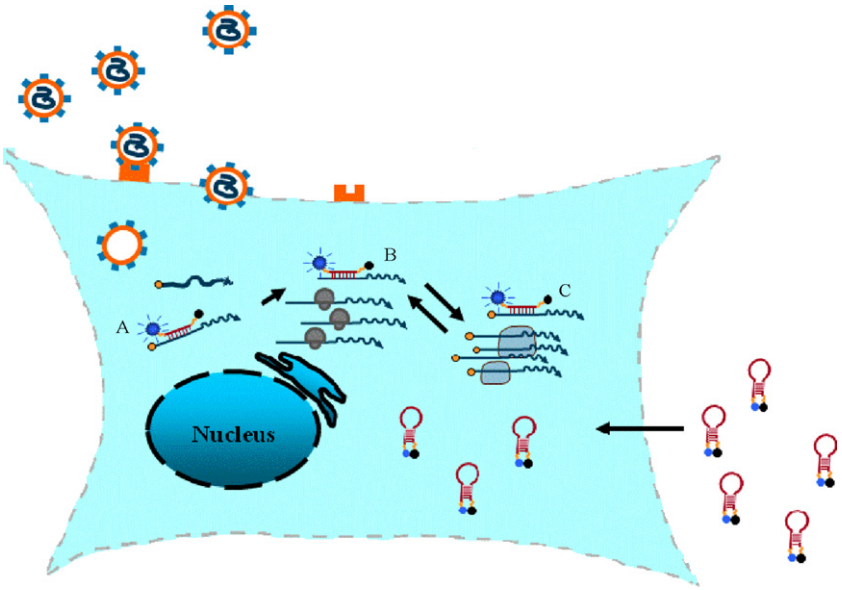

Fig. 1. MBs report the presence of picornavirus by visualizing the fluorescent hybrids with viral RNAs under the fluorescence microscope during the course of viral reproduction, such as: (A) uncoating of viral genome, (B) RNA translation associated with ribosomes (gray) and (C) RNA synthesis on the surface of infected-cell-specific membrane vesicles.

activity" that does not follow the pathways of endocytosis or exocytosis [50]; the penetration across the cell membrane and localize in the cytoplasm and nucleus through an energy-independent mechanism and do not lose their cargo delivery properties when covalently or non-covalently attached to other molecules [51,52]. The peptide-based delivery does not interfere with either specific targeting or hybridization-induced fluorescence of the MBs [53]. TAT peptides have received attention as possible vectors for the delivery of hydrophilic drugs and oligonucleotides for gene therapy or other biological applications. This novel delivery method, when combined with nuclease-resistant MBs, could provide a powerful means for rapid detection and real-time monitoring of viral replication in living cells with high specificity and sensitivity.

Several researchers reported that the introduced oligonucleotides via microinjection or with the help of streptolysin O tend to migrate to the nucleus and this nucleus sequestration affects the cytoplasmic target binding [34,54–56]. In contrast, some studies suggest that the MBs delivered into the cells with the help of streptolysin O and cell penetrating peptides reside within the cytoplasm [52,57]. The pathway that these oligodeoxyribonucleotides probes follow for entry into the cell is still unclear and there is no fundamental biological reason why the probes accumulate in the cell nucleus. Ideally, the intracellular delivery should result in a homogenous distribution after probes being introduced into the cells without interfering with either specific targeting or hybridization-induced fluorescence of the probes. The homogenous distribution of probes within the nucleus and cytoplasm will facilitate the study of different viruses with multiple replication and assembly strategies within different cellular compartments in their viral reproductive cycles.

In addition to probing intracellular RNA synthesis during viral replication by the use of MBs, other viral replication events inside a host cell can be exploited for non-invasive detection. In particular, different genetically engineered cell lines have been established to probe this process in a non-invasive manner. Several viral-inducible reporter systems have been engineered in the host cell for viral detection based on transcription from viral promoters that are specific for virus-infected cells [58,59]. These transgenic cell lines provide a high level of sensitivity and specificity to facilitate the detection process. Unfortunately, this strategy is not applicable for enteroviruses, which exhibit no defined viral promoter region. Many viruses, such as picornaviruses, retroviruses, and caliciviruses, however, produce a polyprotein that is cleaved into individual proteins by virus-specific proteases [60]. Viral protease is a logical target for the detection of infectious viruses because the cleavage event proceeds in a defined manner and is ubiquitous within various viral families. For these viruses, the RNA genome is translated immediately into a single polypeptide upon infection, which is subsequently cleaved by viral proteases to generate mature proteins. This proteolytic process occurs with 100% efficiency and high specificity [61]. Furthermore, proteases are diffusible proteins and can act in the *cis* as well as in the *trans* form in the infected cells. This proteolytic step serves as a good candidate for viral detection because these proteases are highly expressed at an early stage of infection and the proteolysis is extremely efficient and selective.

A simple way to monitor this proteolytic event inside a host cell is to engineer a fluorescent protein pair linked by the target peptide sequence of the protease; proteolysis can be detected based on changes in the fluorescence resonance energy transfer (FRET). FRET is a phenomenon in which energy is transferred from an excited fluorophore, the donor, to a light-absorbing molecule, the acceptor, located within close proximity (typically within 10 nm) (Fig. 2) [62]. Because of the extreme sensitivity of the efficiency of energy transfer from the donor to the acceptor molecule, high resolution FRET imaging has proven to be a valuable means for studying protein–protein interaction as well as the proteolysis of viral replication in living cells [63,64]. Recently, a FRET reporter cell line expressing a hybrid fluorescent indicator composed of a linker peptide, which is exclusively cleaved by the 2A protease ($2A^{pro}$), flanked with a cyan fluorescent protein (CFP) and a yellow fluorescent protein (YFP) allowed the rapid detection (within 7.5 h) of low numbers of infectious enteroviruses (10 PFU or fewer) [64]. In addition, the fluorescence protein pair can be used to probe the dynamic distribution of enterovirus protease in living cells [63]. Although most of these analyses have been performed using fluorescence microscopy to evaluate FRET in the areas of interest, flow cytometry has recently been used to provide automated analysis of fluorescent cells for rapid detection of viral infection [65,66]. The success of the above methods is dependent on the development of stable clone expressing the fluorescent substrate for each protease. An alternative is to deliver a synthetic FRET substrate with the linker peptide with specific proteolytic site for each protease into living cell. Successful application of such an approach was reported recently for *in vivo* measurement of cysteine protease calpain [67]. FRET substrate for the protease was modified with cell penetrating peptide, heptaarginine at the C-terminal.

While the above *in vivo* techniques demonstrate the real-time monitoring of infectious viruses, the success of these methods requires a living cell system. However, many viruses that cause human gastroenteritis, such as Norwalk virus, adenovirus, and astrovirus, cannot be grown in cell culture or grown poorly. The investigation and development of new cell lines for these epidemiologically important waterborne is a clear first challenge but the urgency cannot be overemphasized as the success in adapting non-culturable viruses to grow in cell culture will allow assessment of the viral replication cycle and the consequent understanding of the biology and epidemiology of these viruses [17]. Such knowledge could lead to new strategies for designing and screening drugs against viral infection. Furthermore, real-time molecular detection methods can be combined with the cell culture for rapid detection of infectious viruses and to monitor the progress of viral infection. More sophisticated probes for *in vivo* applications must be able to reduce background in visualizing probe-target hybridization events, to convert target recognition directly into a measurable signal, and to track the multiple steps concerning the produc-

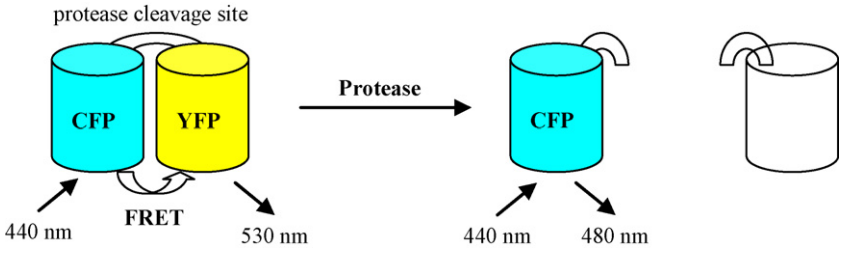

Fig. 2. Schematic representation of fluorescent indicator for monitoring viral proteolytic processing in the infected cells. Detection of infectious viruses will be indicated by changes in FRET (adapted from Ref. [64]).

tion, localization, and transport of specific viral genome during the course of infection.

4. Recent development in the field of nanotechnology for viral detection

MBs and fluorescence protein substrates described above could be readily applied to real-time imaging of gene expression and to study the complexity of viral infection in living cells. A limitation of these molecular probes is the use of organic fluorophore and quencher combinations. The organic fluorophores exhibit low quantum yield and are not suitable for time-lapse microscopy or long-term analysis due to their rapid photobleaching [63,64]. Furthermore, the narrow excitation bands and broad emission bands of the organic dyes cause the spectral overlap and simultaneous light-emission of different probes limit their applications to multiplexing.

Nanotechnology, a field of science that manipulates and utilizes materials on an atomic and molecular scale, generally those less than 100 nm in size, has drawn a growing interest in biological applications for early and specific viral detection [68]. Research on inorganic semiconductor nanocrystals, quantum dot (Qdot), has evolved rapidly on biotechnological and cell-imaging applications. Qdots are colloidal particles consisting of a semiconductor core, a high band gap material shell, and typically an outer coating layer. The core-size-dependent photoluminescence (PL) with narrow emission bandwidths that span the visible spectrum and the broad adsorption spectra allow simultaneous excitation of mixed Qdot populations at a single wavelength. Qdots also exhibit several unique features: high quantum yield, high resistance to photodegradation, and better near-infrared (NIR) emission. Research has shown that the brightness and photostability of Qdots make single-molecule observation over long time scales possible [69]. The simultaneous multicolor approach to single-laser excitation and limited spectral overlap, which improves sensitivity, makes Qdot an attractive alternative to conventional methods in biological detection. Simultaneous excitation of several emission-tunable Qdot populations can be combined with a pool of differentially labeled probes for multiplex target analyses [70–73]. The large absorption window of Qdots paired with the narrow excitation spectra of acceptor dyes significantly reduces unwanted direct excitation of the acceptor and permits only minimal spectral crosstalk between the donor and acceptor emissions, giving near-zero background [74]. These characteristics of Qdots in combination with a multicolor flow cytometer were used by Chattopadhyay et al. for studying the phenotype of multiple antigen specific T-cells [75].

Conjugation of Qdots with organic quenchers like 4-((4-(dimethylamino)phenyl)azo)benzoic acid (DABCYL) or Iowa Black, brings another issue due to their lower quenching efficiency; especially for dyes emitting at longer wavelengths [76]. This may cause problems when different Qdots are employed for simultaneous detection of multiple targets. These non-fluorescent quenchers may not absorb energy properly from the excited state of Qdot thus resulting in higher fluorescence background. Research has shown that the emission of Qdots is effectively quenched by contact with gold nanoparticles as a result of DNA hybridization [77]. One can envision the potential use of Qdots and gold nanoparticles as FRET pairs will improve the detection limits and expand the potential applications of FRET-based molecular probes [78].

5. Conclusion

Viruses will always remain our major health threat, and the inclusion of techniques described above call for the development of multiplex approaches with the aim to detect and characterize several pathogens in a single assay, including the rapidly evolving old and new viral pathogens. Principles must be applied to fulfill the aforementioned goals such as the qualitative diagnosis and the accurate quantitative determination to enable prospective virological safety approach based on the identification of viral pathogens. Furthermore, an important aspect of the present method is to provide insight into the molecular mechanisms underlying the virulence of the highly health-significant viruses for future efforts aiming at the development of antiviral treatments.

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