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Universal virus detection by degenerate-oligonucleotide primed polymerase chain reaction of purified viral nucleic acids

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A B S T R A C T

This study describes a novel non-specific universal virus detection method that permits molecular detection of viruses in biological materials containing mixtures of cells and viruses. Samples are subjected to nuclease digestion and ultracentrifugation to separate encapsidated viral nucleic acids from cellular nucleic acids. A degenerate oligonucleotide primer PCR (DOP-PCR) that has been optimized for the non-specific amplification of virus sized genomes is then employed. Virus identification is performed by sequencing of cloned DOP-PCR products followed by sequence comparison to sequences published in GenBank.

This method was used to detect a variety of DNA viruses (including HSV, VZV, SV40, AAV, and EBV) and RNA viruses (including HTLV-I, HTLV-II, influenza, and poliovirus), which were spiked into cells, constitutively expressed in cell culture, or detected in productively infected cultured cells. This novel approach was compared with a non-specific virus detection method used previously and found to be several logs more sensitive. This type of approach has potential utility in solving virus detection and discovery problems where other methods have failed.

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1. Introduction

The detection of unknown viruses is still one of the most challenging problems in diagnostic virology. If a known virus is suspected to be present in a biological sample, it is usually not difficult to use the known viral sequence to design specific PCR primers, and to implement a specific detection assay. However, in an era of emerging pathogens and bioterrorism (including the potential for bioterrorism agents genetically engineered to avoid detection using standard methods), improved methods for virus discovery and detection that are less dependent upon the availability of sequence information are needed. Moreover, if such methods were standardized, they could be used potentially to demonstrate (to a defined degree of sensitivity) the absence of a contaminating virus in a biological specimen with potential application to clinical or regulatory problems.

Conventional PCR methods are used frequently to identify or exclude the presence of a viral pathogen suspected to be present in a biological sample. PCR is both very sensitive and specific, especially when coupled with sequencing of PCR products. However, its reliance on specific primers complementary to the pathogen genome sequence makes conventional PCR analyses unsuitable for screening of biological samples for the presence of unknown viruses.

PCR methods using primers based on consensus sequences shared among related viruses were used to detect parainfluenza viruses 1–3 (Corne et al., 1999), to identify and detect coronaviruses (Sampath et al., 2005; van der Hoek et al., 2004), to discover new herpes viruses (VanDevanter et al., 1996) and to identify West Nile virus as the etiologic agent of human encephalitis in the recent New York outbreak (Briese et al., 1999). These primers are designed to anneal to sequence regions highly conserved among members of a virus family, such as polymerase genes. Because these regions are almost never completely conserved, these consensus primers generally include some degeneracy that permits binding to all or the most common known variants on the conserved sequence. This general approach has been automated in an innovative high throughput system (Sampath et al., 2005). However, to be success-

Abbreviations: DOP-PCR, degenerate oligo primer-polymerase chain reaction; RDA, representational difference analysis; CPE, cytopathic effect; SISPA, sequence-independent single primer amplification.

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ful, these methods require sufficient sequence information on a group of related viruses to permit the design of consensus primers.

Subtractive methods can identify genomic sequence differences between two related samples, and have also been used for virus discovery. Representational difference analysis (RDA) (Lisitsyn et al., 1993) involves molecular subtraction of uninfected negative control sequences from cells believed to also contain virus. Differential display also involves the comparison of amplified representational libraries of two or more different samples. RDA was used to discover HHV-8 (Chang et al., 1994) and TTV (Linnen et al., 1996), while differential display was used to first identify human metapneumovirus growing in infected cell cultures (van den Hoogen et al., 2001). Even though these methods are not limited generally by primer specificity, they require a matched uncontaminated negative control that is otherwise identical genetically to the test sample, and they are technically challenging and time consuming.

The goal of the present study was to develop sensitive molecular techniques that could be used to detect viruses rapidly in biological specimens, in the absence of any specific information about the virus and without the need for a negative sample. In order to identify viral genomes in any given biological sample, it is necessary to use methods that exclude cellular nucleic acids, which are the most abundant nucleic acids in most samples. The approach described here is based on physical separation of viral and cellular nucleic acids, followed by non-specific amplification of the purified viral nucleic acids. Encapsidation of viral nucleic acids is used as a basis for separation of viral nucleic acids from cellular nucleic acids. Viral capsids protect viral nucleic acids from digestion by nucleases, and also provide viral nucleic acids with a density that permits capsids to be separated from other cellular debris, including nucleic acids (Denniston et al., 1981). After purification of viral capsids, viral DNA and RNA are extracted (separately, in order to preserve an indication of whether a viral genome is DNA or RNA) and RNA is copied into cDNA by reverse transcription. Samples are then amplified using a non-specific DOP-PCR assay that is designed to amplify at least a portion of any viral genome. This report discusses this method and an evaluation of its sensitivity to detect viruses spiked into cell culture, present during acute infection of cell culture, or expressed constitutively in cell culture.

2. Materials and methods

2.1. Samples

Samples used to assess the sensitivity of the non-specific virus detection methods included virus stocks, Vero cells (African Green Monkey Kidney Cells) spiked with varying amounts of virus stocks, cells known to be infected chronically or latently with various viruses (either alone or spiked into uninfected Vero cells), and cells acutely infected with known DNA or RNA viruses (either alone or spiked into uninfected Vero cells). SV40 genomic DNA (obtained from Life Technologies, Gaithersburg, MD), in 10-fold dilution series, was used as a control.

2.1.1. Cells

Vero, 1A2 (Croce et al., 1979), MJ (G11) (Popovic et al., 1983), MoT (Saxon et al., 1978), and Jiyoye (Kohn et al., 1967) cells were sourced from the American Type Culture Collection (Manassas, VA), and maintained in culture according to the instructions provided. To investigate detection of viruses expressed in cells, 1A2, MJ, MoT and Jiyoye cells were harvested and pelleted by centrifugation, resuspended in medium, counted and then diluted serially (10-fold serial dilutions containing 10^6 to 10^1 cells), using uninfected Vero cells to make up a total of 10^6 cells.

2.1.2. Viruses

HSV-1 (strain 17syn+) (Dr. Stephen Straus, NIH), HSV-2 (strain 333) (Dr. Gary Hayward, Johns Hopkins University), influenza type A virus (Dr. Zhiping Ye, CBER/FDA), titered stocks of simian virus 40 (SV40) (Dr. Andrew Lewis, CBER/FDA), varicella-zoster Virus (Oka strain) (Michiaki Takahashi, Osaka University, Japan), poliovirus type 2 (Sabin strain) (Dr. Konstantin Chumakov, CBER/FDA) and adeno-associated virus (AAV type 2) (Dr. Robert Kotin, NIH) were used in these experiments.

2.1.3. Cell spiking experiments

Viruses were diluted serially in cell culture medium to obtain 10-fold serial dilutions of 10^6 to 10^1 infectious units. Virus dilutions were mixed with 10^5 or 10^6 uninfected Vero cells.

2.1.4. Infected cell experiments

HSV-1 and HSV-2 were used to infect Vero cells at a multiplicity of infection (MOI) of 1. Human poliovirus type 2 was used to infect semi-confluent Vero cells at a MOI of 1. Varicella-zoster virus (VZV) was grown in MRC-5 cells. Cells were harvested when about 75% cytopathic effect (CPE) was evident. The cells were pelleted by centrifugation, resuspended in medium, and counted using a hemocytometer. Virus-infected cells were serially diluted to obtain 10-fold serial dilutions of 10^6 to 10^1 infected cells. Infected cell samples were then adjusted to a total of 10^6 cells using uninfected Vero cells.

2.2. Purification of viral nucleic acids

The viral nucleic acid extraction method is based on viral capsid purification techniques described previously (Denniston et al., 1981). Briefly, samples were suspended in viral buffer (30 mM Tris/HCl pH 7.5, 3.6 mM $CaCl_2$, 5 mM Mg Acetate, 125 mM KCl and 0.5 mM EDTA), and homogenized (using a disposable tip homogenizer, Omni International, Marietta, GA) to disrupt cell and nuclear membranes. Cellular nucleic acids were digested away by treatment with nucleases DNase I (100 Kunitz Units, Sigma–Aldrich, St. Louis, MO) and RNase ONE (50–100 U, Promega, Madison, WI). Samples were then extracted with 0.4 volumes of trichlorotrifluoro-ethane (Sigma–Aldrich, St. Louis, MO). The encapsidated viral nucleic acids were recovered in the aqueous phase and then pelleted through a discontinuous glycerol gradient as described previously (Denniston et al., 1981). The capsid pellet was resuspended in viral buffer and was re-treated (as above) with DNase and RNase. Viral nucleic acids (DNA and/or RNA) were extracted from the isolated capsids using the Qiagen (Valencia, CA) Whole Blood Kit (for DNA) or the Trizol reagent (Invitrogen, Carlsbad, CA) (for RNA). In order to detect viral RNA, samples were subjected to reverse transcription using random hexamer primers and Superscript Reverse Transcriptase (Life Technologies, Gaithersburg, MD) prior to DOP-PCR.

2.3. Degenerate oligonucleotide primer polymerase chain reaction (DOP-PCR)

A method described previously for generating representational libraries of eukaryotic genomes, known as degenerate oligonucleotide primer PCR (DOP-PCR) (Telenius et al., 1992) was adapted to amplify non-specifically portions of any viral genome. DOP-PCR primer populations were designed with a short (four to six nucleotide) 3′-anchor sequence (which allows the primers to bind in consistent locations), preceded by a non-specific degenerate sequence (of six to eight nucleotides in these experiments). Immediately upstream of the non-specific degenerate sequence, each