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Use of a universal virus detection assay to identify human metapneumovirus in a hematopoietic stem cell transplant recipient with pneumonia of unknown origin

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Abstract

Background—Development of uncommon viral infections in immunocompromised transplant recipients can pose major diagnostic challenges. We present a case report of an immunocompromised patient suffering from pneumonia, for which the causative agent was not identified by routine methods.

Objectives—To identify the potential cause of the pneumonia using a degenerate oligonucleotide primer (DOP) PCR assay which is designed to detect all viruses.

Study Design—DOP-PCR was applied to bronchoalveolar lavage fluid from this patient. Generic PCR products were cloned and sequenced.

Results—The novel universal virus assay detected human metapneumovirus in the clinical sample. The finding was confirmed by two independent metapneumovirus specific PCRs targeting independent regions of the viral genome.

Conclusions—The DOP PCR was used to detect and identify the sequence of an unidentified virus. This study provides proof of concept for the use on clinically relevant specimens of this unbiased universal assay, which requires no previous viral sequence information.

Keywords

Virus discovery; virus detection; immunocompromised host; virus disease diagnosis; virus disease etiology

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Introduction

Detection of unknown viruses remains one of the most challenging problems in virology. When classical methods fail to detect viruses, development of molecular tests depends upon the availability of accurate sequence information. Generic virus detection tests allow for the simultaneous detection of multiple (if not all) virus sequences. We previously developed and optimized an improved universal virus detection method that sensitively and broadly detects DNA and RNA viruses in infected cell cultures and spiked cells with limiting amounts of nucleic acid or viruses¹. This technique requires no prior sequence information from the virus being detected, implying promise for virus discovery and detection of virus in samples for which other methods have failed.

A case of pulmonary infiltrates and fever of unknown origin occurring in a hematopoietic stem cell transplant (HSCT) recipient was investigated using a modified version of the generic virus detection assay after conventional methods failed to identify a pathogen. Preceding studies of bronchoalveolar lavage (BAL) fluids were negative for typical respiratory pathogens using standard microbiological techniques, including bacteria (including nocardia, legionella, mycoplasma, chlamydia and mycobacteria), fungi, and pneumocystis. Routine viral studies for influenza A and B, adenovirus, parainfluenza, herpes simplex virus, cytomegalovirus, and respiratory syncytial virus were also negative. An aliquot of the previously tested BAL was obtained after it was stored at refrigerator temperatures for several days. Because the storage conditions might have damaged virus capsids (and thus made the viral nucleic acid vulnerable to nuclease digestion) we modified the previously described purification procedure¹ by eliminating the use of nucleases.

This method is based on physical separation of host and virus nucleic acids followed by a degenerate oligonucleotide primer PCR (DOP-PCR) that is optimized for virus-sized genomes and permits non-specific nucleic acid amplification at a high degree of sensitivity (100-1000 copies of viral nucleic acid). The PCR uses a single primer population with a short conserved 3' sequence (TGTGG) and is several logs more sensitive than a previously described non-specific viral PCR assay². The DOP-PCR was experimentally shown to amplify a range of viral genomes, including the DNA viruses SV40, HSV, VZV, EBV, and adeno-associated virus, and the RNA viruses poliovirus, influenza virus, HTLV-1, and HTLV-2¹.

Materials and Methods

Research was deemed exempt by the Offices of Human Subjects Research at NIH and performed in accordance with the ethical standards of FDA and NIH institutional review boards, and with the Helsinki Declaration of 1975, as modified in 1983. Two 200 μ L BAL aliquots were centrifuged at $18,000 \times g$ for 2 hrs at 4°C. DNA and RNA were separately extracted from 100 μ L of each supernatant and pellet (AllPrep DNA/RNA kit, Qiagen, Valencia, CA). The cDNA syntheses were carried out using a first strand kit and random hexamer primer (Invitrogen, Carlsbad, CA). DOP-PCR was carried out separately on DNA and cDNA purified from one BAL aliquot in a reaction containing 1.5 mM $MgCl_2$, 10 mM KCl, 10 mM Tris pH 8.4, 200 μ M dNTP, 2.4 μ M DOP primer (5'-CCGACTCGAGINNNNNNTGTGG-3') and 2.5 U Low DNA Taq (Applied Biosystems, Foster City, CA); cycling conditions were 5 min at 95°C; 5 cycles of 1 min at 94°C, 1.5 min at 30°C, slow ramping to 72°C at 0.2°C/sec, and 3 min extension at 72°C; and 35 cycles of 1 min at 94°C, 1 min at 55°C, and 2 min extension at 72°C, with the addition of 14 seconds per cycle to each extension step. DOP-PCR products were analyzed and purified by gel electrophoresis (results for cDNA are shown in Figure 1a). Four individual bands were purified from the cDNA amplification and ligated into a TOPO-TA cloning vector and transformed into competent bacteria (Invitrogen, Carlsbad, CA). DOP-PCR of DNA extracted from the

BAL yielded a non-discrete smear consistent with non-specific amplification of cellular nucleic acids, and was not analyzed further. Sequencing was performed using a 3130xl Genetic Analyzer (Applied Biosystems, Foster City, CA). Sequences from the obtained clones were compared with the non-redundant (nr) database in GenBank using TBLASTX (NCBI, Bethesda, MD).

Primer sequences for HMPV specific PCR were: N-gene forward primer: GGYRACYTTTRCTWAARGAATCATCAGG; N-gene reverse primer: CAGATTCRGGRCCTATYTCTC; L-gene forward primer: CTTGAGATGGTATTAAATGA; L-gene reverse primer: CCTACACTTAATTCTCTTTC; PCR cycling conditions were 94°C for 2 min, 40 cycles of 94°C 20 sec, 55°C 1 min, 72°C 1 min followed by 10 min 72°C.

Results

We sequenced DNA from four bands representing DOP-PCR products derived from cDNA. Five out of 63 clones from the DOP-PCR yielded sequence that matched the known sequence of the G-gene from human metapneumovirus (Figure 1b). The largest band (~620 bp) yielded human mitochondrial sequence, the second band (~480 bp) yielded two human mitochondrial sequences and two HMPV sequences, the third band (~420 bp) yielded one human sequence, and the smallest band (~250 bp) yielded four human and three HMPV sequences. The HMPV sequences were all derived from the G-gene, with some overlap. Other clones contained vector sequences only.

To confirm the presence of HMPV in this sample, duplicate BAL samples were subjected to independent PCRs targeting nucleoprotein and polymerase gene genomic regions. Primer sequences were chosen (with some degeneracy) to permit detection of all seven HMPV full length genomes in GenBank. The RT-PCRs for the nucleoprotein gene tested positive for two independent supernatant and pellet samples each, while the L-gene (polymerase) sequence was only detected in the pellet but not in the supernatant samples, implying lower sensitivity for this test (Figure 1c). The relative locations of the DOP-PCR and specific PCR products are shown in Figure 1d.

Discussion

The detection of HMPV sequences in the clinical sample does not prove conclusively that this virus was the sole cause of the pneumonia; reports indicate that viral sequences may persist for long periods of time in murine lung tissue³. Detected HMPV sequences could represent either viable or non-viable virus particles. However, we detected signals in supernatant as well as in pellets, implying that both virus capsids and free viral nucleic acid were present. The presence of readily-detectable HMPV sequences in BAL fluid using the generic DOP assay as well as two independent PCRs for different regions of the genome imply a high probability of very recent or ongoing HMPV infection in this individual.

We identified different but overlapping DOP-PCR products in the HMPV G-gene, and did not detect other HMPV genes. The HMPV sequence is not biased in a way that would support preferential DOP-PCR amplification of the G-gene; thus, it seems more likely that the sample contained more RNA from the G-gene than from other genes. This may have been related to the refrigerator storage of the sample for five days prior to analysis—conditions that may have caused a significant amount of RNA degradation.

Although identification of HMPV in clinical samples is usually not a major diagnostic challenge, genetic heterogeneity among HMPV strains can lead to false negative RT-PCR assays, leading some investigators to seek further improvement in these assays^{4,5}. Our

findings imply that the DOP-PCR based universal virus assay is useful for finding viruses in patient samples where other methods have failed to identify a pathogen. This can be particularly important when sample quantity is limited, making it difficult to perform many additional tests. A single generic assay that is capable of detecting all DNA and RNA viral genomes, paired with an appropriate purification technique, thus has significant advantages. The major strength of the DOP assay is its ability to amplify viral sequences without prior knowledge of those sequences, increasing the likelihood of finding mutated or novel viruses or viruses with substantial sequence heterogeneity, including as-yet undiscovered, unexpected, or non-human viruses. This is of major importance because most (about 75%) emerging infectious diseases and 80% of select agents are of zoonotic origin⁶.

Unlike many subtraction-based generic approaches, the non-subtractive DOP assay does not require a non-infected but otherwise genetically identical control. As compared with amplification methodologies using primers based on consensus sequences shared among related viruses^{7,8,9} (e.g., those that were used to discover new herpes viruses¹⁰ and to identify West Nile virus as the etiologic agent of human encephalitis in the New York outbreak¹¹), the DOP assay does not require the performance of multiple assays directed at different virus families, which may be impractical when sample is limited. The DOP assay also does not require accurate sequence information for each virus included in the screening¹² in order to establish the assay, as do consensus primer and microarray-based techniques. Other recently described alternatives to classical virological methods used to identify a new arenavirus¹³, a virus linked to colony collapse disorder of bees¹⁴ and a polyomavirus playing a role in human Merkel cell carcinoma¹⁵ use a combination of degenerate PCR and high-throughput sequencing, an approach that can be expensive and is not readily available. Enrichment of viral nucleic acids in the present approach permits identification of viral sequences even by traditional sequencing, making this approach accessible to laboratories with access to PCR, cloning, and standard sequencing. The DOP-PCR assay requires three days from sample receipt to sequence.

The DOP-PCR approach is most promising in situations where sample is limited, suspicion of viral disease is high, and standard viral diagnostic tests are negative. Viruses with substantial genetic heterogeneity and those that are not initially suspected based on the clinical presentation would be particularly amenable to detection using this method.

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Abbreviations

DOP-PCR

degenerate oligonucleotide primer PCR

HSCT

hematopoietic stem cell transplant

BAL

bronchoalveolar lavage