Mini Review

Use of Suppression Subtractive Hybridization for Viral Diagnostics

Mohammed M. Islam[1,3]*, Damian F. J. Purcell[2] and George Kannourakis[1,3]

[1]Fiona Elsey Cancer Research Institute, 106-110 Lydiard Street South, Ballarat Technology Park Central, Ballarat, VIC 3353, Australia; [2]Department of Microbiology and Immunology, Peter Doherty Institute for Infection and Immunity, The University of Melbourne, Parkville, Victoria 3010, Australia; [3]School of Applied and Biomedical Sciences, Federation University, Victoria, 3350, Australia.

Abstract | Efficient identification of viruses is paramount to not only define the cause of uncharacterized infections but also to estimate the dynamics of viral population in the infected hosts. Several recent methodologies and chemistries have been proposed for viral diagnostics with variable outcomes and successes. In this review, we discuss a novel approach to identify the least transcripts of viral genomes using suppression subtractive hybridization that can be adapted to unanimously identify viruses of diverse genetic backgrounds.

Editor | Muhammad Munir, The Pirbright Institute, UK.
Received | May 01, 2016; **Accepted** | May 10, 2016; **Published** | May 12, 2016
***Correspondence** | Mohammed M. Islam, School of Applied and Biomedical Sciences, Federation University, Victoria, 3350, Australia; **Email:** monirul@fecri.org.au.
DOI | http://dx.doi.org/10.17582/journal.bjv/2016.3.2.41.46
Citation | Islam, M.M., D.F.J. Purcell and G. Kannourakis. 2016. Use of suppression subtractive hybridization for viral diagnostics. *British Journal of Virology*, 3(2): 41-46.
Keywords: SSH, Virus, Diagnostics, Infection, MHV

The dynamic and sensitive identification of disease-causing viruses is crucial to understand mechanisms of virus pathogenesis, characterization of unknown viruses and to devise strategies for the control and therapeutics. Identifying complex combinations of viruses, responsible for causing known or unknown infections, is problematic because diagnostic virology used in mainstream medical practice is highly directed towards finding the "usual suspects" (Leland and Ginocchio, 2007; Read et al., 2000; Storch, 2000; Tang and Ou, 2012). Viral diagnostics are generally grouped into three main categories: **(i)** direct detection, **(ii)** indirect examination (i.e. virus isolation), and **(iii)** serology. In direct detection, the clinical specimen is examined directly for the presence of virus particles, virus antigen or viral nucleic acids. In indirect examination, the specimen is introduced into cell culture, embryonated eggs or animals in an attempt to grow the virus, commonly referred as virus isolation. A serological detection method involves the detection of rising titers of virus specific antibody between acute and convalescent stages of infection.

Direct detection methods are rapid (results are available within 24 hours) and include determination of characteristic morphology of known viruses, and direct antigen or nucleic acid identification. The electron microscopy (EM) has insufficient sensitivity and the specificity for scarce viral particles in clinical samples without virus amplification by other methods including cultivation, isolation and propagation. Beside these complications, viral cultivation is a highly selective step that might overlook unknown or interacting mixtures of viruses. Direct immunofluorescence of specific viral antigen can also provide results within few hours (Landry et al., 1997; Landry et al., 2000),

but again poor sensitivity requires virus cultivation that may limit the recovery of novel viruses that resist culture. Viruses with extensive antigenic heterogeneity that lack cross-reacting antigens are unlikely to be detected by antibody-detection techniques (Landry and Ferguson, 2000).

Viral nucleic acid detection method, also known as molecular method, is the most reliable method among direct detection methods (Niesters, 2004). Classical molecular techniques such as dot-blot and southern blot depend on the use of specific DNA/RNA probe for hybridization. The specificity and sensitivity of these techniques are slightly better than the antigen detection method. On the other hand, newer molecular techniques such as polymerase chain reaction (PCR), ligase chain reaction or branched DNA (bDNA) depend on some form of amplification, either the target or the signal itself, and are far more sensitive methods. As a result, these techniques have become the key procedures in most, if not all, virus diagnostic laboratories (Bej et al., 1991; Wagar, 1996; Wolcott, 1992). Amongst all molecular methods, PCR is an extremely sensitive method and is capable of identifying as small as single DNA molecule in a clinical specimen. Moreover, the method is relatively fast and can be finished within very short turnaround time. Hence, PCR has become the most popular diagnostic method for viral infection (Emmanuel, 1993; Fan et al., 1998; Harrison, 1998). Introduction of multiplex PCR has sped up the diagnosis process, as well as reduced the expenses (Elnifro et al., 2000; Grondahl et al., 1999). However, cross contamination is a major risk for the PCR detection method but with improved and automated nucleic acid sample isolation techniques this risk has been substantially reduced. As the PCR system depends on a primer design, only specific viruses or viruses with high sequence homology with the target viruses can be identified using this technology.

Virus isolation was once considered the 'gold standard' to concentrate virus for detection but its importance has diminished with the technological advancement such as development of monoclonal antibodies or introduction of molecular diagnostics, that have provided powerful specific tools to detect the presence of viral infection (Hsiung, 1984). However, virus isolation still remains necessary because it is the only technique capable of providing viable isolate that can be used for further characterization, such as phenotypic antiviral susceptibility testing (Storch, 2000). Virus culture may permit the expansion and detection of multiple viruses, although it may be difficult to determine their relative importance in the clinical samples.

Serology forms the traditional method of viral diagnosis. Following exposure to viral antigen, the adaptive immune system of vertebrate sequentially produces different isotypes of virus specific antibodies that bind to the virus and render it non-infectious mainly through neutralization. Firstly, IgM is produced for a few weeks before B-lymphocytes switch to produce high titers of IgG that can be sustained indefinitely with levels amplified rapidly upon a secondary infection. The presence of IgM in the blood is an indicator of acute infection whereas IgG indicates that an infection occurred previously. The clinical utility of serology is limited by the need for comparison of acute and convalescent antibody titers or the detection of virus specific IgM.

Many different types of serological tests are available but are limited to the available viral antigen panels. The sensitivity and specificity of serological assays depend greatly on the antigen, and recombinant protein or synthetic peptide antigens tend to be more specific than those using whole or disrupted virus particles (Van Regenmortel, 1993). Serology, like other viral diagnostic methods, has a number of associated problems. For example, **(i)** a longer time required for diagnosis for paired acute and convalescent sera, **(ii)** extensive antigenic cross-reactivity between related viruses e.g. HSV and VZV (Takayama, 1994; Vafai et al., 1990), Japanese encephalitis and dengue viruses (A-Nuegoonpipat et al., 2008; Yamada et al., 2003) may lead to false positive results, **(iii)** immunocompromised patients often give a reduced or absent humoral immune response and **(iv)** patients given blood or blood products may give a false positive result due to the transfer of antibodies.

Taken together, from the above discussions, it is evident that none of the currently known viral diagnostic methods are well tuned to identify novel viruses. It is also evident that a known viral morphology or antigenicity, antibody specificity or specific molecular probes or primer sequences are necessary to identify a putative virus. A virus that does not show any of the known characteristic viral features, as mentioned above, cannot be identified using currently available viral diagnostic methods. For example, we encountered a situation where a novel retrovirus was suspect-

ed to be involved in the infection of human histiocytosis cells that were xenografted into SCID mice after direct EM analysis of disease tissue (Ristevski et al., 1999). Serological analysis was not possible, as these immunocompromised mice did not raise antibody to the infecting agent. Virus isolation was also failed reasons that are currently not known. PCR methods using target degenerate primers also failed to identify the virus (Islam et al., 2015). At that stage, we used suppression subtractive hybridization (SSH) to successfully identify the putative virus, MHV-MI (Islam et al., 2015). SSH is a molecular biology method, which to our knowledge, has not been previously used in viral diagnostics.

Originally, SSH was used to compare two populations of transcripts (mRNAs) and to obtain clones of genes that are only expressed in one population, irrespective of their relative abundance in that population (Diatchenko et al., 1996). The basic principle of the method involves the conversion of both mRNA populations to cDNAs, hybridization of the cDNAs to each other, removal of the hybridized cDNAs and then amplification of the unhybridized or subtracted cDNAs by suppression PCR. Rare transcripts are amplified in the last suppression PCR step and hence provide nearly perfect subtraction results. SSH leads to fewer false positives compared to the other techniques used to identify differentially expressed genes (Adam et al., 2012; Lukyanov et al., 2007; Sahebi et al., 2014). This method has been successfully used to identify rare transcripts or exclusively expressed genes in an estrogen receptor-positive breast carcinoma cell line (Kuang et al., 1998), in the liver of patients with chronic hepatitis C virus infection or liver cirrhosis (Patzwahl et al., 2001; Shackel et al., 2003), in metastatic melanoma cell line (Patzwahl et al., 2001), and in lung adenocarcinoma (Wu et al., 2013). We have, for the first time, reported that SSH can be used for the identification of unknown viruses from an infected tissue (Islam et al., 2015).

In our experimental SSH, we extracted total RNA from both infected and non-infected mice liver and then converted the mRNA populations of the total RNA into cDNAs using reverse transcriptase. The cDNAs from infected mouse liver were called 'tester-cDNAs' and that from non-infected mouse liver were called 'driver cDNAs'. A forward subtraction was completed as per the procedure described (Islam et al., 2015) using tester cDNAs and driver cDNAs. Subtracted cDNAs were then cloned into *E.coli* using pGEM vector and made into a forward subtracted cDNA library. A total of 148 positive clones from the subtracted cDNA library were isolated and their cloned DNAs were sequenced. Twelve percent (12%) of the library was identified to contain murine hepatitis sequences. Rest of the sequences belonged to murine genes (Table 1). SSH method clearly identified a murine hepatitis virus, which was initially unknown and couldn't be identified by a serological or molecular method. SSH method has definitely solved the problem of identifying the virus that was involved in SCID mice liver infection.

Table 1: *Gene list from forward cDNA subtraction*[†]

HITS	Percentage	Gene Name
18	12	Murine Hepatistis Virus Nucleocapsid, membrane proteins etc.
14	9	Mouse serum amyloid A (SAA) family proteins
14	9	Mouse fibrinogen (alpha, beta and gamma) polypeptide
6	4	Mouse Annexin A protein family
3	2	Mouse lactotransferine
2	1.33	Mouse serine or cystein peptidase inhibitor (SP1-2) clade member
2	1.33	Mouse mRNA for Ly-6 alloantigen (ly-6 E.1)
1	0.68	89 individual mouse genes

[†]*Table has been reproduced from our earlier publication (Islam et al., 2015)*

SSH is an unbiased method and can be used to identify any viral infection. All viruses produce their transcripts (mRNAs) in the host cells irrespective of their genetic makeup (DNA or RNA virus). Subtraction of total mRNA of non-infected control cells from that of infected host cells (forward subtraction) produces differential mRNA population that contains mRNA population from viral origin. Cloning and sequencing that differential mRNA population gives information about the genetic make-up of the infecting virus. The virus could be a known virus or a completely unknown virus. Full-length genome sequencing of the known virus is often unnecessary but can be done by using virus specific primers. On the other hands, full-length genome sequencing of unknown virus is crucial for the complete identification of the virus and it is a difficult task. A sequence identity search of the cloned cDNA (partial genome) in the DNA databank

gives an impression about the putative virus or its origin (genus). A virus with the highest identity may be a close relative of the putative virus. If the putative virus is a DNA virus (single stranded or double stranded), genetic material can be isolated from the host cell and then directly used for sequencing. If the putative virus is a RNA virus, cDNA of the virus can be synthesized from the total RNA of the host cell and then sequenced using designed sequencing primers, which may not necessarily be 100% identical to the target virus sequences. In our SSH findings, putative virus had the highest homology (95 -97%) with an RNA virus, murine hepatitis virus (MHV) and we designed the sequencing primers from the genomic sequence of MHV-A59 virus. Since MHV is single stranded RNA virus, we prepared cDNA from the host cell (infected liver cells) and then sequenced the whole genome using a total of 45 pairs of designed primer. Nearly 70% of the full-length viral genome was sequenced using 23 pairs of designed primer from MHV-A59 genomic sequence and rest of the sequence was identified using nested primers and gene walking strategy. 3'-end of the viral genome was amplified using 3'-RACE and then when sequenced. 5'-RACE did not work, so we used 5'-end consensus primer for sequencing 5'- end.

Although the basic principle of the SSH method looks very simple, it is actually a lengthy process. More than one month is necessary to complete the whole process. Steps involved in the method are: (1) isolation of total RNA from infected and non-infected tissue samples, (2) preparation of cDNA from both RNAs, (3) Digestion of cDNA using *Rsa* I restriction enzyme, (4) purification of the digested cDNA, (5) ligation of adaptors to driver cDNA, (6) hybridization of tester and driver cDNA, (7) Suppression PCR, (8) purification of PCR products, (9) cloning PCR products into host vector system, (10) preparation of the subtracted cDNA library, (11) isolation of cDNA clones, and (12) sequencing number of clones needed for statistical significance. Some of the above mentioned methods are very crucial and need to be quality tested before going to the next step. Hence additional time is necessary to complete the process. The largest amount of time (nearly one month) is taken by the steps 9 – 12, whereas only one week is necessary to carry out the actual subtraction procedure (steps 1-8). If, somehow, we could avoid or bypass steps 9-12, we could substantially reduce the time needed. Adopting next generation sequencing at step 9 might give similar results but we haven't tested the possibility yet.

Differential RNA sequencing between the infected and non-infected subjects could also be used as alternative for SSH, but the risk of losing rare transcripts is very high as no suppression PCR is involved in RNA sequencing. In fact, viral transcripts would be rare compared to the infected host cell transcripts and may not be identifiable by RNA sequencing. Besides this, differential RNA sequencing produces relative (comparative) abundance of transcripts but the absence of transcript in the non-infected cells could be problematic for bioinformatics and may not be suitable for detecting viral infection. Hence, to our understanding, SSH is the only unbiased method, which can be effectively used for the detection of a completely unknown virus in infected tissue or specimens. Another advantage is that this process also selects host genes that are expressed in response to the viral infection. The only drawback is the length of time required to complete the technique. However, introducing some automation in different steps or replacing steps 9-12 with next generation sequencing can easily reduce the time required to complete the process to nearly one week. As a result, we are proposing that SSH could become an ideal technique for detection of rare viral infection.

A recent variation on the SSH technique is the VirCapSeq method that uses millions of synthetic oligonucleotides that are prepared against every known vertebrate viral sequence, including polymorphic nucleotides (Briese et al., 2015). This technique can also greatly enrich viral sequences 10,000 times for direct deep sequence analysis. However, again it is limited to identifying only the known viruses whose sequence has contributed to the design of the selection probes. Perhaps using these probes to deplete SSH libraries will enhance the identification of completely new viral entities.

References

- Adam, N.M., Sorooshain, S., Zhang, X., Tezara, C., Azizi, P., Shahebi, M., Bande, Y., Nurhaiza, S., Kaiser, A., 2012. Comparison of suppression subtractive hybridization with other methods used to identify differentially expressed genes in plants. In: Adam, N. M., Sorooshian, S. (ed). Engineering Research Methods. Pp. 15-24. Lulu.com, Serdang, Malaysia.
- A-Nuegoonpipat, A., Panthuyosri, N., Anantapreecha, S., Chanama, S., Sa-Ngasang, A., Sawan-

panyalert, P. and Kurane, I. (2008). Cross-reactive IgM responses in patients with dengue or Japanese encephalitis. *J Clin Virol* 42: 75-77. http://dx.doi.org/10.1016/j.jcv.2007.10.030

- Bej, A.K., Mahbubani, M.H, Atlas, R.M. (1991). Amplification of nucleic acids by polymerase chain reaction (PCR) and other methods and their applications. *Crit Rev Biochem Mol Biol* 26: 301-334.
- Briese, T., Kapoor, A., Mishra, N., Jain, K., Kumar, A., Jabado, O.J., Lipkin, W.I. (2015). Virome Capture Sequencing Enables Sensitive Viral Diagnosis and Comprehensive Virome Analysis. *MBio* 6: e01491-15. http://dx.doi.org/10.1128/mBio.01491-15
- Diatchenko, L., Lau, Y.F., Campbell, A.P., Chenchik, A., Moqadam, F., Huang, B., Lukyanov, S., Lukyanov, K., Gurskaya, N., Sverdlov, E.D., Siebert, P.D. (1996). Suppression subtractive hybridization: a method for generating differentially regulated or tissue-specific cDNA probes and libraries. *Proc Natl Acad Sci U S A* 93: 6025-6030. http://dx.doi.org/10.1073/pnas.93.12.6025
- Elnifro, E.M., Ashshi, A.M., Cooper, R.J., Klapper, P.E. (2000). Multiplex PCR: optimization and application in diagnostic virology. *Clin Microbiol Rev* 13: 559-570. http://dx.doi.org/10.1128/CMR.13.4.559-570.2000
- Emmanuel, P.J. (1993). Polymerase chain reaction from bench to bedside. Applications for infectious disease. *J Fla Med Assoc* 80: 627-630.
- Fan, J., Henrickson, K.J., Savatski, L.L. (1998). Rapid simultaneous diagnosis of infections with respiratory syncytial viruses A and B, influenza viruses A and B, and human parainfluenza virus types 1, 2, and 3 by multiplex quantitative reverse transcription-polymerase chain reaction-enzyme hybridization assay (Hexaplex). *Clin Infect Dis* 26: 1397-1402. http://dx.doi.org/10.1086/516357
- Grondahl, B., Puppe, W., Hoppe, A., Kuhne, I., Weigl, J.A., Schmitt, H.J. (1999). Rapid identification of nine microorganisms causing acute respiratory tract infections by single-tube multiplex reverse transcription-PCR: feasibility study. *J Clin Microbiol* 37: 1-7.
- Harrison, T.J. (1998). The polymerase chain reaction--a time of transition from research to routine. *J Clin Pathol* 51: 491-492. http://dx.doi.org/10.1136/jcp.51.7.491
- Hsiung, G.D. (1984). Diagnostic virology: from animals to automation. *Yale J Biol Med* 57: 727-733.
- Islam, M.M., Toohey, B., Purcell, D.F., Kannourakis, G. (2015). Suppression subtractive hybridization method for the identification of a new strain of murine hepatitis virus from xenografted SCID mice. *Arch Virol* 160: 2945-2955. http://dx-.doi.org/10.1007/s00705-015-2592-y
- Kuang, W.W., Thompson, D.A., Hoch, R.V., Weigel, R.J. (1998). Differential screening and suppression subtractive hybridization identified genes differentially expressed in an estrogen receptor-positive breast carcinoma cell line. *Nucleic Acids Res* 26: 1116-1123. http://dx.doi.org/10.1093/nar/26.4.1116
- Landry, M.L., Cohen, S., Ferguson, D. (2000). Impact of sample type on rapid detection of influenza virus A by cytospin-enhanced immunofluorescence and membrane enzyme-linked immunosorbent assay. *J Clin Microbiol* 38: 429-430.
- Landry, M.L. and Ferguson, D. (2000). SimulFluor respiratory screen for rapid detection of multiple respiratory viruses in clinical specimens by immunofluorescence staining. *J Clin Microbiol* 38: 708-711.
- Landry, M.L., Ferguson, D., Wlochowski, J. (1997). Detection of herpes simplex virus in clinical specimens by cytospin-enhanced direct immunofluorescence. *J Clin Microbiol* 35: 302-304.
- Leland, D.S. and Ginocchio, C.C. (2007). Role of cell culture for virus detection in the age of technology. *Clin Microbiol Rev* 20: 49-78. http://dx.doi.org/10.1128/CMR.00002-06
- Lukyanov, S.A., Rebrikov, D., Buzdin, A.A. (2007). Suppression subtractive hybridization. In: Anonymous , Nucleic Acids Hybridization Modern Applications. Pp. 53-84. Springer, Berlin. http://dx.doi.org/10.1007/978-1-4020-6040-3_3
- Niesters, H.G. (2004). Molecular and diagnostic clinical virology in real time. *Clin Microbiol Infect* 10: 5-11. http://dx.doi.org/10.1111/j.1469-0691.2004.00699.x
- Patzwahl, R., Meier, V., Ramadori, G., Mihm, S. (2001). Enhanced expression of interferon-regulated genes in the liver of patients with chronic hepatitis C virus infection: detection by suppression-subtractive hybridization. *J Virol* 75: 1332-1338. http://dx.doi.org/10.1128/JVI.75.3.1332-1338.2001
- Read, S.J., Burnett, D., Fink, C.G. (2000). Molecular techniques for clinical diagnostic virol-

ogy. *J Clin Pathol* 53: 502-506. http://dx.doi.org/10.1136/jcp.53.7.502

- Ristevski, S., Purcell, D.F., Marshall, J., Campagna, D., Nouri, S., Fenton, S.P., McPhee, D.A., Kannourakis, G. (1999). Novel endogenous type D retroviral particles expressed at high levels in a SCID mouse thymic lymphoma. *J Virol* 73: 4662-4669.
- Sahebi, M., Hanafi, M.M., Abdullah, S.N., Rafii, M.Y., Azizi, P., Nejat, N., Idris, A.S. (2014). Isolation and expression analysis of novel silicon absorption gene from roots of mangrove (Rhizophora apiculata) via suppression subtractive hybridization. *Biomed Res Int* 2014: 971985. http://dx.doi.org/10.1155/2014/971985
- Shackel, N.A., McGuinness, P.H., Abbott, C.A., Gorrell, M.D., McCaughan, G.W. (2003). Novel differential gene expression in human cirrhosis detected by suppression subtractive hybridization. *Hepatology* 38: 577-588. http://dx.doi.org/10.1053/jhep.2003.50376
- Storch, G.A. (2000). Diagnostic virology. *Clin Infect Dis* 31: 739-751. http://dx.doi.org/10.1086/314015
- Takayama, M. (1994). Reactivity of varicella-zoster virus subunit antigens in enzyme-linked immunosorbent assay to sera from varicella, zoster, and herpes simplex virus infections. *Med Microbiol Immunol* 183: 1-11. http://dx.doi.org/10.1007/BF00193626
- Tang, Y.W. and Ou, C.Y. (2012). Past, present and future molecular diagnosis and characterization of human immunodeficiency virus infections. *Emerg Microbes Infect* 1: e19. http://dx.doi.org/10.1038/emi.2012.15
- Vafai, A., Wroblewska, Z., Graf, L. (1990). Antigenic cross-reaction between a varicella-zoster virus nucleocapsid protein encoded by gene 40 and a herpes simplex virus nucleocapsid protein. *Virus Res* 15: 163-174. http://dx.doi.org/10.1016/0168-1702(90)90006-W
- Van Regenmortel, M.H. (1993). Synthetic peptides versus natural antigens in immunoassays. *Ann Biol Clin (Paris)* 51: 39-41.
- Wagar, E.A. (1996). Direct hybridization and amplification applications for the diagnosis of infectious diseases. *J Clin Lab Anal* 10: 312-325. http://dx.doi.org/10.1002/(SICI)1098-2825(1996)10:6<312::AID-JCLA2>3.0.CO;2-8
- Wolcott, M.J. (1992). Advances in nucleic acid-based detection methods. *Clin Microbiol Rev* 5: 370-386.
- Wu, M., Tu, T., Huang, Y., Cao, Y. (2013). Suppression subtractive hybridization identified differentially expressed genes in lung adenocarcinoma: ERGIC3 as a novel lung cancer-related gene. *BMC Cancer* 13: 44-2407-13-44.
- Yamada, K., Takasaki, T., Nawa, M., Yabe, S., Kurane, I. (2003). Antibody responses determined for Japanese dengue fever patients by neutralization and hemagglutination inhibition assays demonstrate cross-reactivity between dengue and Japanese encephalitis viruses. *Clin Diagn Lab Immunol* 10: 725-728. http://dx.doi.org/10.1128/cdli.10.4.725-728.2003