Metagenomics and the Case of the Deadly Hamster

Palacios G, Druce J, Du L, Tran T, Birch C, Briese T, et al. A new arenavirus in a cluster of fatal transplant-associated diseases. N Engl J Med 2008;358:991-998. (Reprinted with permission from the Massachusetts Medical Society.)

Abstract

Background: **Three patients who received visceral-organ transplants from a single donor on the same day died of a febrile illness 4 to 6 weeks after transplantation. Culture, polymerase-chain-reaction (PCR) and serologic assays, and oligonucleotide microarray analysis for a wide range of infectious agents were not informative.** ***Methods:*** **We evaluated RNA obtained from the liver and kidney transplant recipients. Unbiased high-throughput sequencing was used to identify microbial sequences not found by means of other methods. The specificity of sequences for a new candidate pathogen was confirmed by means of culture and by means of PCR, immunohistochemical, and serologic analyses.** ***Results:*** **High-throughput sequencing yielded 103,632 sequences, of which 14 represented an Old World arenavirus. Additional sequence analysis showed that this new arenavirus was related to lymphocytic choriomeningitis viruses. Specific PCR assays based on a unique sequence confirmed the presence of the virus in the kidneys, liver, blood, and cerebrospinal fluid of the recipients. Immunohistochemical analysis revealed arenavirus antigen in the liver and kidney transplants in the recipients. IgM and IgG antiviral antibodies were detected in the serum of the donor. Seroconversion was evident in serum specimens obtained from one recipient at two time points.** ***Conclusions:*** **Unbiased high-throughput sequencing is a powerful tool for the discovery of pathogens. The use of this method during an outbreak of disease facilitated the identification of a new arenavirus transmitted through solid-organ transplantation.**

Comment

The article by Palacios and colleagues[1] documents three transplant recipients who shared the same donor and succumbed to a novel arenavirus infection. Similar events occurred with the very recent infection of two kidney recipients by lymphocytic choriomeningitis virus (LCMV) from a single donor this year,[2] and this complements a report from 2006 detailing other transplant recipients dying from LCMV infection.[3] In the latter LCMV clusters, the timing of infection and viral phylogenetic analysis left little doubt that the infections were derived from a point source.[3] Even though there was no evidence of LCMV infection in the two donors, both suffered hemorrhagic intracranial lesions consistent with LCMV infection, and one owned an LCMV-infected pet hamster.[3,4] The report by Palacios and colleagues is particularly notable because of the methods used to discover the novel arenavirus. Their exhaustive search started with standard culture techniques for viruses and bacteria and progressed to extensive polymerase chain reaction (PCR) studies using primers complementary to over 30 known viral and bacterial families.[1] The authors then tried pan-microbial microarray analysis with 29,455 oligonucleotide probes reactive to known vertebrate viruses, bacteria, fungi, and parasites,[5] and after drawing a blank with all these studies, they resorted to brute sequencing of all RNA in the infected tissue to discover the new virus.[1] This report brings up important issues concerning the role of rodent-derived pathogens in transplant patients. There is also a great deal of interest in the high-throughput sequencing methods that were used to detect the new arenavirus as well as the arrival of metagenomics as the new kid on the block for viral discovery.

Metagenomics is defined as the sequenced-based analysis of the collective microbial genomes contained in an uncultured environmental sample.[6] The approach has been used for multiple purposes, including the cataloging of ocean microbial communities[7] and the identification of a viral pathogen responsible for honey bee colony collapse.[8] In humans, metagenomic analysis has been used to study viral communities in blood and respiratory secretions,[6] to differentiate bacterial species in gut flora,[9] and to catalog the collective DNA and RNA viral species in stool samples of healthy subjects[10,11] and patients with diarrhea.[12] American, European, and Asian consortia are now aligned to conduct studies in humans focused on bacteria in the gut and other body cavities,[13] and metagenomics has emerged as the methodology of choice for virus discovery in humans over the last year.[12,14] For example, Merkel cell carcinoma is found in immunosuppressed individuals, and this suggests an infectious etiology. Therefore, investigators at the University of Pittsburgh sequenced ~380,000 transcripts extracted from the rare skin cancer.[14] After biocomputational processing, 2 of the ~2400 nonhuman sequences were found to share homology with polyomaviruses. Subsequently, sequences from the newly identified Merkel cell polyomavirus were detected in 80% of tumor samples and 5% of controls.[14] In essence, the number needed to sequence

(NNS) in order to discover the novel Merkel cell polyomavirus was 1/190,000, which appears cost-prohibitive for current capillary sequencing. In contrast, Palacios and colleagues[1] sequenced ~94,000 transcripts from infected allografts and found 14 sequences that resembled an Old World arenavirus, providing an NNS of approximately 1/6700 for their viral agent. Both groups used high-throughput pyrosequencing, which is far cheaper than capillary sequencing (Fig. 1).

Will the brute-force approach of high-throughput sequencing catch on for virus discovery? The answer is undoubtedly yes. The process is unbiased, relatively insensitive to a low copy number, lacks the inherent problem with PCR of widespread contamination, and has the potential to provide a complete representation of all viruses within the environment.[15] On reflection, even Houghton's team, cloning hepatitis C virus (HCV) with state-of-the-art molecular complementary DNA cloning techniques in the 1980s, eventually had to screen more than 20 million individual clones before they finally identified a gene product reactive to anti-HCV.[16] They would have saved a considerable amount of time and effort if they could have sequenced RNA from a few "non-A non-B virus" infected livers (assuming that they could have had access to human genome data that were not available at the time). However, there are additional considerations, such as the copy number of the virus in infected liver samples and the predicted NNS required to detect the agent. Clearly, the other major issue with metagenomics is the cost.

With the marked reduction of sequencing costs predicted in the near future, high-throughput sequencing will become more popular. Ironically, most of this technology development is being driven by the global efforts to identify genetic determinants of human disease.[17] An international consortium of American, British, and Chinese researchers is promising to sequence 1000 human genomes in the next 3 years.[18] To put these developments in perspective, when one of us (G.K.-S.W.) sequenced the rice genome in 2002,[19] most of the major genome centers were producing high-quality "finished" sequences at $0.10 per base pair. With the latest technologies, however, a similar product can now be created for approximately $0.001 per base pair. The new instruments are quite different from the ones used by the original human genome

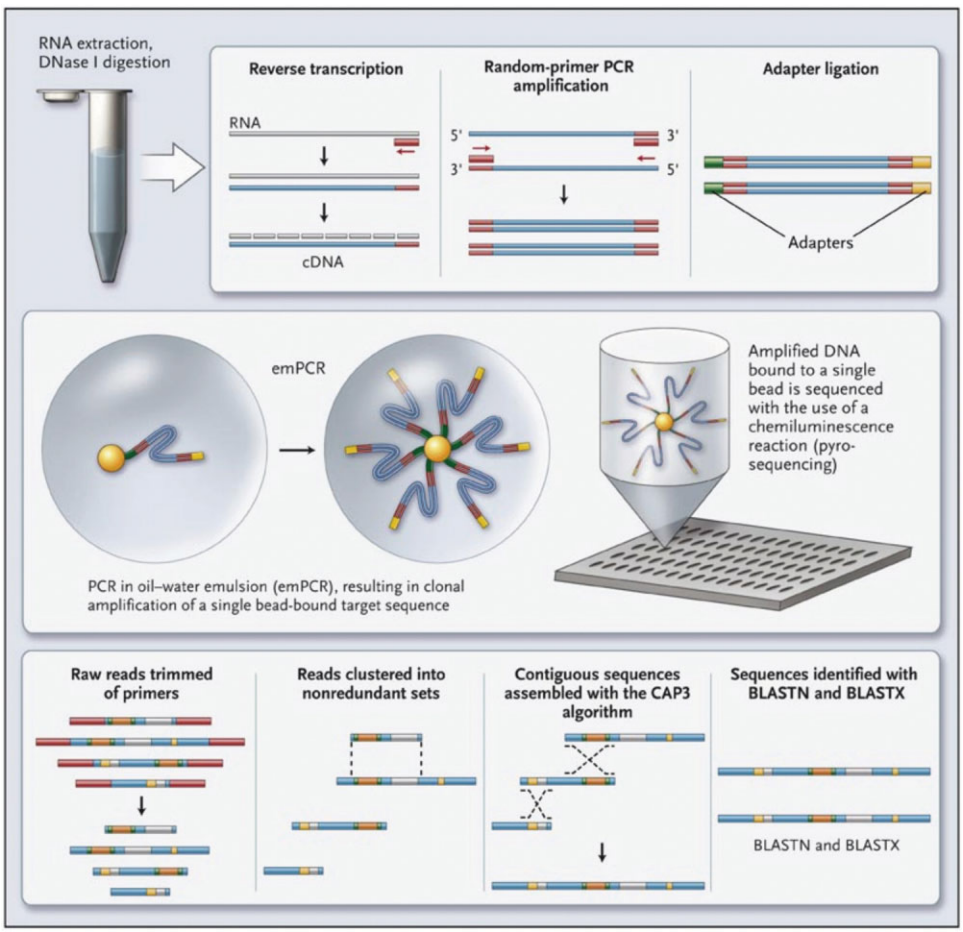

Fig. 1. High-throughput pyrosequencing method used by Palacios et al.[1] to identify the novel arenavirus. Abbreviations: cDNA, complementary DNA; emPCR, emulsion polymerase chain reaction; PCR, polymerase chain reaction. Reprinted with permission from the *New England Journal of Medicine*.[1] Copyright 2008, Massachusetts Medical Society. All rights reserved. See reference 34 for review of donor screening.

project, insofar as they do not use electrophoresis to separate the DNA molecules. Instead, they capture single DNA molecules on a solid support, amplify them *in situ*, perform sequence-dependent enzymatic reactions, and then detect the outcomes to determine sequence content (Fig. 1). Given the highly competitive nature of this industry, continuing advances in sequencing technology and falling costs are to be expected. Accordingly, it is just a matter of time before metagenomics becomes a practical research technique for determining bacterial and viral contents of clinical samples without the need to culture.[15]

Let us go back to the plot: what do we know of the novel arenavirus found by Palacios and colleagues? The answer is very little, apart from the fact that the 57-year-old male donor died from a cerebral hemorrhage 10 days after returning to Australia after a 3-month visit to rural regions of former Yugoslavia.[1] Genetically, the agent shares close homology with other Old World arenaviruses, such as LCMV and the Lassa virus, that use rodents as the main reservoir of infection. Lassa virus is usually passed from rodent excreta in grain and can also be transmitted from infected patients to healthcare workers. Although no wide-ranging Lassa virus epidemics have occurred, zoonoses from mice cause 5000 deaths from hemorrhagic fever in Western Africa per year.[20] Depending on the location, a variable proportion (~10%) of the population has serologic evidence of prior Lassa virus infection with a calculated ratio of infection to fatality of approximately 1% to 2%.[21] In North America, the seroprevalence of LCMV is approximately 5%, and the estimated fatality in LCMV-infected individuals is much less than 1%, but the mortality is far greater in immunosuppressed and otherwise vulnerable populations.[3,22] LCMV infection is associated with nonspecific symptoms such as fever, myalgia, and headache, and viremia is limited to the second week of infection in immunocompetent individuals. Infrequently, a second phase of disease occurs after convalescence with meningitis and infection disseminated to other organs.[3,22]

Therefore, to what extent should we be worried by rodent infections in our transplant patients, and should we be screening donors for these emerging pathogens? We know that about 60% of emerging infectious diseases in humans occur as a result of zoonoses.[23] While it is rare for zoonotic viruses to adapt to become epidemic in humans, there are notable exceptions, such as the severe acute respiratory syndrome (SARS) virus epidemic that arose as a result of a zoonosis from bats.[24] On occasion, a zoonosis may become permanently resident in the human population as endemic infection, as observed with human immunodeficiency virus I and human immunodeficiency virus II derived from chimpanzees and sooty mangabeys, respectively.[25] Thankfully, the occurrence of fatal rodent-borne infections in our transplant population is rare. However, is it avoidable? Currently, there are no Food and Drug Administration–approved diagnostic tests for LCMV or the newly discovered arenavirus. Moreover, the testing of donors by serology or PCR may not be cost-effective or helpful in detecting an acute infection with a limited window of viremia.[22] Thus, measures to limit the spread of viruses may provide a degree of protection. Pet hamsters have been implicated in LCMV outbreaks,[4,22] and infection in expectant mothers has been linked to intrauterine death and congenital LCMV infection with severe neurological consequences in the newborn.[26] As a result, our options for controlling the spread of rodent viruses to transplant recipients may be limited to ensuring that our pets are free of known human pathogens.[22] Indeed, an outbreak of monkeypox in the Midwestern United States from pet rodents[27] underscores the need for implementation of tighter regulations to ensure the absence of zoonotic viruses in pet stores.[22]

Currently, a detailed donor history is the only screening methodology that we have to identify the potential for donor transmission of infectious agents that cannot be readily detected in serum by Food and Drug Administration–approved diagnostic methods (Table 1). To prevent LCMV transmission, for example, some organ procurement agencies have taken an

Table 1. Donor Screening for Infectious Agents in North America[34]

Screening by Laboratory Testing
Hepatitis B virus surface antigen and hepatitis B core antibody
Hepatitis C virus antibody; HCV RNA*
Cytomegalovirus IgG antibody
Epstein-Barr virus antibody panel
HIV I and II antibody; HIV RNA*
Human T-cell lymphotrophic virus I and II antibody
Treponema pallidum rapid plasma reagin or syphilis enzyme immune assay
Toxoplasma gondii antibody
West Nile virus nucleic acid testing†
Trypanosoma cruzi serology†
Screening by Medical History
Mycobacterium tuberculosis (symptoms, past medical, family, and exposure history)
Rabies virus (symptoms, recent animal bite)
Lymphocytic choriomeningitis virus† (symptoms, rodent exposure, including new pets within 6 weeks)
Creutzfeldt-Jakob disease (symptoms, past medical history, recipient of human-derived pituitary growth hormone or dura mater)

Abbreviations: HCV, hepatitis C virus; HIV, human immunodeficiency virus; IgG, immunoglobulin G.

*High-risk donors for recent transmission of infection.

†Regional or seasonal testing or patients with relevant travel history.

approach similar to that adopted for avoidance of West Nile virus infection, in which donors with encephalitis, meningitis, or flaccid paralysis of undetermined etiology are deferred.[28] An inquiry regarding rodent exposure, such as the acquisition of a new pet within the preceding 6 weeks, may identify potential and generally asymptomatic LCMV infection in a prospective donor. Unlike West Nile virus, which has a specific geographic distribution and a seasonal variance, LCMV is endemic in rodents and therefore has no predictable pattern. This presents a real problem, as mice are prevalent in most households and are detected in up to 95% of residences, especially in kitchens.[29] We may not have appreciated the full impact of rodent infections on human disease beyond their obvious contribution to dramatic disease outbreaks such as plague, typhus, and hanta virus.[25] More recently, several mouse viruses have been implicated in chronic human disorders, including the endogenous mouse retroviruses, which can be expressed to become infectious agents in other species. For example, the mouse mammary tumor virus, a murine betaretrovirus, has been linked to human breast cancer[30] and primary biliary cirrhosis.[31,32] Mouse gammaretroviruses have also been identified in human samples, such as the xenotrophic murine leukemia virus (MuLV)–related virus found in human prostate cancer[33] and MuLV sequences found in normal gut flora detected by metagenomic analysis.[11] Although the diverse roles of these agents in human disease have yet to be determined and remain a source of controversy, further metagenomic studies are likely to tell us the diversity of rodent microbial agents that can be found in humans. Until then, additional vigilance and a detailed donor history for rodent exposure will be required to prevent the introduction of zoonotic infections into our transplant recipients.

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A Yellow Bullet Against the Drivers of Hepatic Fibrogenesis

Sato Y, Murase K, Kato J, Kobune M, Sato T, Kawano Y, et al. Resolution of liver cirrhosis using vitamin A-coupled liposomes to deliver siRNA against a collagen-specific chaperone. Nat Biotechnol 2008;26:431-442. (Reprinted by permission from Macmillan Publishers Ltd: Nature Biotechnology, copyright 2008. www.nature.com/nbt/index.html.)

Abstract

There are currently no approved antifibrotic therapies for liver cirrhosis. We used vitamin A-coupled liposomes to deliver small interfering RNA (siRNA) against gp46, the rat homolog of human heat shock protein 47, to hepatic stellate cells. Our approach exploits the key roles of these cells in both fibrogenesis as well as uptake and storage of vitamin A. Five treatments with the siRNA-bearing vitamin A-coupled liposomes almost completely resolved liver fibrosis and prolonged survival in rats with otherwise lethal dimethylnitrosamine-induced liver cirrhosis in a dose- and duration-dependent manner. Rescue was not related to off-target effects or associated with recruitment of innate immunity. Receptor-specific siRNA delivery was similarly effective in suppressing collagen secretion and treating fibrosis induced by CCl_4 or bile duct ligation. The efficacy of the approach using both acute and chronic models of liver fibrosis suggests its therapeutic potential for reversing human liver cirrhosis.

Comment

Progressive liver fibrosis, or scarring of the liver, as the common consequence of all chronic liver diseases is a major medical and economic challenge, because there are no effective treatment options except liver transplantation. The current paradigm attributes this fibrogenic response to hepatic stellate cells (HSCs), which reside in the subendothelial space of Disse and are the major storage site for vitamin A in healthy humans. In response to injury, HSCs undergo an activation process in which they lose vitamin A, become highly proliferative, and synthesize abnormal extracellular matrix, including excessive collagen, various proteoglycans, and structural glycoproteins.[1] This activation process and the deposition of extracellular matrix in the space of Disse are commonly considered to represent the key pathogenetic events in liver fibrosis.[2]

Previous studies showed that the collagen-specific chaperone heat shock protein 47 (HSP47)—also termed J6, gp46, collagen-binding 48 kDa protein (CB48), collagen binding protein 2 (CBP2), or colligin—plays an essential role in regulating collagen synthesis. It is constitutively expressed with collagens and binds to both helical and nonhelical forms of collagens.[3] The link between *Hsp47* and collagen production was demonstrated in *Hsp47* knockout mice that are severely deficient in the mature, propeptide-processed form of α(I) collagen and fibril structures in mesenchymal tissues.[4] Simultaneous activation of both type-I and type-III collagens with *Hsp47* expression was also reported in HSCs during carbon tetrachloride (CCl_4)-induced liver fibrosis in rats.[5] Conversely, the constitutive overexpression of *Hsp47* promotes the secretion of collagens in vascular smooth muscle cells.[6] As a molecular chaperone, HSP47 binds closely to procollagen in the endoplasmic reticulum, but dissociates from it in the Golgi complex to allow triple helix formation.[7]

Based on these properties *Hsp47* has been suggested as plausible molecular target to interfere with fibrogenesis (Fig. 1). Initial proof-of-concept was obtained in experimental models, demonstrating that small interfering RNA (siRNA) targeting *Hsp47* inhibited the expression of type-I collagen and the formation of scar tissue.[8,9]

The study by Sato et al.[10] targets siRNA directed against *Hsp47* to HSCs. The authors used a key feature of HSCs, that is, uptake and storage of vitamin A, highlighted by Hans Popper more than 60 years ago.[11] Sato et al. prepared retinol-coupled liposomes carrying siRNA against *Hsp47* and demonstrated binding of retinol binding protein (RBP) to these liposomes (via gel filtration), receptor-mediated uptake of the RBP-coupled complexes by HSCs in vitro (via flow cytometry), and suppression of cellular collagen secretion (via Sirius red binding assays).

Using three different models of liver fibrosis (dimethylnitrosamine [DMN], CCl_4, and bile duct ligation), the study assessed the antifibrotic properties of the siRNA-carrying liposomes *in vivo* at comparatively low doses of 0.75 mg/kg body weight. As shown by immunofluorescence and fluorescence-activated cell sorting analysis of isolated liver cells, the liposomes were predominantly