

Is the Era of Viral Culture Over in the Clinical Microbiology Laboratory?

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Conventional tube culture systems have long been the mainstay in clinical virology for the growth and identification of viruses from clinical specimens. Innovations such as centrifugation-enhanced shell vial and multiwell plate cultures and the use of genetically engineered and mixed cell lines, coupled with faster detection of viral replication, have allowed for reasonable turn-around times for even some of the most slowly growing clinically important human viruses. However, molecular methods, in particular, the PCR, have usurped the role of viral culture in many laboratories, limiting the use of this traditional method of virus detection or replacing it altogether. Advances and improvements in molecular technology over time have also resulted in newer generations of more rapid and accurate molecular assays for the detection, quantification, and genetic characterization of viruses. For this point-counterpoint, we have asked two individuals, Richard L. Hodinka of the Children's Hospital of Philadelphia, a clinical virologist whose laboratory has completely eliminated viral culture in favor of molecular methods, and Laurent Kaiser, head of the Virology Laboratory at the University of Geneva Hospital, who continues to be a strong advocate of viral culture, to discuss the relevance of viral culture in the molecular age.

POINT

In the fast-paced medical world of wanting or needing an immediate and accurate diagnosis, viral culture has lost its place and relative importance in diagnostic virology. To me, as a director of a busy hospital-based clinical virology laboratory, there is nothing more disheartening than to invest considerable time, labor, and resources in test systems that yield less-than-adequate results in a less-than-timely fashion. In such a setting, the impact on clinical care and patient management is diminished and the overall effectiveness of the laboratory is called into question by those who use its services. Within the past 3 decades, we have experienced a significant change in the diagnostic landscape with the development of a variety of molecular technologies designed to rapidly and accurately detect, differentiate, and quantify many different viruses of medical importance. Led by real-time PCR (1, 2, 3), applications of these molecular methods appear endless and their use is now commonplace in larger academic medical centers and tertiary-care facilities and is expanding into smaller laboratories and even to the point of care as simpler and more accessible testing platforms come to market.

Viral culture—why bother? Culture-based systems for virus isolation (4) have been the “gold standard” in clinical virology for decades and have served the laboratory well when there was little else available for the diagnosis of viral illnesses. However, there are many legitimate reasons why the use and relative importance of virus culture are declining with the continued development of rapid and accurate molecular tests. In general, the isolation of viruses in culture is slow, time-consuming, and labor-intensive and lacks the sensitivity needed to have an appreciable impact on clinical decision making. Many clinically relevant viruses are simply difficult to grow or cannot be grown at all in cultured cells, while other viruses require specialized culture systems that are either not available or too complicated for routine use in diagnostic laboratories. Traditional tube cultures, although viewed as being comprehensive in growing a wide range of viruses and capable of detecting unsuspected new viruses or more common viruses in new places, fail to isolate viruses in many instances and can take

days to weeks to provide a final result. While centrifugation-assisted cultures using individual, mixed, or genetically engineered cell lines are designed to be faster and more user-friendly than tube cultures, they are not always as sensitive and are normally limited by the quality and availability of reagents and the number and types of cell lines that can be used to grow a variety of different viruses. Normally, only viruses that are being sought after or for which the cell lines are designed can be identified and only one or a few viruses can be detected at a time. Also, waiting an average of 1 to 2 days or longer for centrifugation-assisted culture results delays clinical decision making and is no longer necessary when using molecular methods. Typically, the growth and identification of viruses in culture require specialized facilities and considerable expertise; however, experience is diminishing and becoming more variable with time and it is increasingly more difficult for many clinical virology laboratories to maintain their proficiency and competency with culture-based systems. Lastly, viral culture systems really have not been standardized or scrutinized to the same extent as molecular testing and can vary considerably, depending upon the selection of appropriate cell lines; the adequate collection, transport, and handling of specimens to ensure virus viability; and the maintenance of viable and healthy inoculated cells.

It has been argued that there will always be special circumstances where viral culture is needed in clinical virology. This may be true at some level, but not necessarily at the point of primary care, where the relative importance of culture has changed. Molecular technology, when used directly on clinical specimen ma-

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terial, can be just as effective, if not more so, in yielding a variety of different viruses, whether suspected or not; providing proof of active infection and possibly disease causation; linking virus strains to defined outbreaks; identifying emerging or novel viral pathogens; and performing drug susceptibility testing. When needed for defined reasons, the isolation of viruses could be done by select local, regional, or national laboratories that maintain culture systems and the expertise necessary to use them. Such laboratories could also maintain biorepositories of archived viruses for future characterization and use if desired.

Molecular methods—why not? Without question, we are witnessing one of the most exciting and dramatic revolutions in clinical laboratory medicine, particularly in the diagnosis of viral diseases. The accuracy and timeliness of viral diagnosis have been vastly improved with the continuous development and implementation of molecular amplification methods. The overall performance of molecular assays is exceptional and far exceeds that of virus culture, and an ever-growing number of viruses can now be readily detected, differentiated, and quantified by PCR (1, 2, 3) and other molecular amplification technologies (5), such as nucleic acid sequence-based amplification, strand displacement amplification, transcription-mediated amplification, branched DNA amplification, loop-mediated amplification, and helicase-dependent amplification. Molecular methods are now becoming the new gold standard and rapidly displacing traditional culture-based procedures in many laboratories. Real-time PCR has reached the greatest maturity over the years and has proven to be simple, fast, highly sensitive and specific, reproducible, cost-effective, and versatile to perform. This system has truly transformed viral diagnostics in my laboratory. All of our 37 real-time PCR-based assays, which include both qualitative and quantitative testing formats, can be routinely performed every day, 7 days a week, and some are done multiple times a shift over one or two shifts. With real-time PCR, we can do more in a single day than ever imaginable with any culture-based system and can readily incorporate new tests into our work flow. It has been many years since we have done viral cultures, and our health care providers have not missed them.

Need we debate the clinical utility of molecular testing? The advantages of molecular testing for viral diagnosis and monitoring are now widely recognized (1, 6). Qualitative molecular assays allow the early detection of viruses prior to the development of a detectable immune response or when it may be more difficult or impossible to grow the virus in culture or detect it by rapid antigen tests. An early and accurate diagnosis can have a prompt and significant impact on patient care by providing timely treatment that may limit the extent of disease and reduce associated sequelae and by reducing or eliminating unnecessary hospitalization, diagnostic procedures, inappropriate use of antimicrobial agents, and associated costs. A specific diagnosis can also help to prevent or reduce hospital spread of viruses and provide a better understanding of viruses circulating in the community and can be beneficial when screening donated blood to help improve the safety of the blood supply.

Molecular methods can detect viruses for which existing tests are considerably less accurate or for which no tests exist. The technologies are being used successfully to detect uncultivable, fastidious, or slow-growing viruses and to detect viruses that are new or otherwise too dangerous to grow. Molecular amplification methods are especially well suited for detecting viruses present in small specimen volumes or that are in low numbers or nonviable within

clinical specimens. Multiplex procedures have been developed and commercialized for the simultaneous detection of multiple viruses from a single specimen (7) when more than one virus can cause similar or identical clinical manifestations. Quantitative molecular amplification assays have become invaluable tools to assess disease progression and prognosis, monitor therapy, predict treatment failure and the emergence of drug resistance, and facilitate our understanding of the transmission and pathogenesis of certain viruses in chronically infected and immunocompromised hosts (8–11). Genotypic assays involving nucleic acid sequencing are being used for the recognition of genetic variants that may be resistant (12–14) or refractile (9) to antimicrobial drugs and can provide useful information about the role of genetic diversity for vaccine development and efficacy, the evolution and phylogenetic relationships among closely related viruses, the pathogenic and epidemiological behavior of viruses, the interplay between viruses and their hosts, and virus discovery (15).

Molecular testing is not all a bed of roses. Adopting molecular testing is not without challenges. There are concerns expressed by many colleagues that molecular testing is still not for the majority of clinical laboratories. Issues of assay availability and accessibility remain; laboratory-developed assays and analyte-specific reagents are still commonly used and require considerable expertise, time, and equipment and must undergo extensive verification and validation. Quantitative molecular assays are still highly variable and require additional standardization (16). Technically, there are concerns about false-negative results due to PCR inhibitors and viral genetic diversity, false positives due to contamination, detection of latent infections or viruses from asymptomatic individuals, and the clinical significance of viral coinfections and the persistence of viral signals beyond the acute presentation of clinical disease. There is a definite need for commercial products and platforms and simpler technologies that all can use; more assays continue to come down the pipeline, and some have been licensed by the Food and Drug Administration, but few manufacturers have broad menus that meet the demands of expanded applications. Laboratories also continue to struggle to overcome budgetary constraints and space limitations and the need for highly trained personnel. The addition of molecular testing is normally viewed as an increase in laboratory costs, particularly if traditional testing is not replaced. However, the use of molecular methods may actually lead to an overall cost savings for the laboratory and institution and should be carefully analyzed. Lastly, reimbursement for molecular testing must be sufficient but varies by state and depends upon payer mix and capitation rates.

Advances in molecular technology. More recently, molecular testing has rapidly evolved and is being downsized and simplified beyond our wildest imaginations. The field of nanotechnology is growing by leaps and bounds and being aggressively applied to molecular diagnostics (17, 18). Advances in microelectronics, microfluidics, and microfabrication have paved the way for new technologies and ever simpler molecular platforms with the ultimate goal of sample-in/answer-out testing for all laboratories regardless of size, resources, or capacity.

Miniaturization and simplification of highly complex molecular procedures are now a reality. With the introduction of biochips, microarrays, nanoparticles, nanobiosensors, and nanopores, we are seeing more high-performance, easy-to-use, specimen-to-result, multiplex molecular platforms for broad-based viral detection, syndrome-specific panel testing, and detection of genetic variants and

drug resistance genes. This should extend the availability of molecular diagnostics to every laboratory and even to the point of care (18) and will shape the future of clinical virology and infectious disease testing for years to come. Coupling of PCR with electrospray ionization mass spectrometry (19) has even led to a commercial platform for universal pathogen detection, identification, strain typing, and determination of virulence and resistance genes where the organism's identity does not need to be anticipated.

Conclusions. When all is said and done, it is difficult to envision how viral culture will continue to have diagnostic relevance given the ever-changing and -improving world of molecular technology. Only modest improvements in culture-based systems have been made over many years, and they are simply not enough for sustainability in this century and beyond. Today, molecular methods have led to discoveries never before imagined when using culture-based methods. We are now capable of performing unparalleled molecular studies of virus-host interactions to provide remarkable amounts of new clinical information about our virome and the more global microbiome, the viral resistome, the human transcriptome, the pharmacogenomics of antiviral therapy, and the phylogenomics of viral evolution. The future holds great promise for the continued advancement of molecular technology in the clinical virology laboratory and an even greater impact on the care and management of patients.

Richard L. Hodinka

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COUNTERPOINT

I recently visited the brand-new clinical virology laboratory of a large reference university hospital that was equipped with the ultimate technology and the most modern molecular tools, including next-generation sequencing platforms dedicated to routine investigations, and had a staff of outstanding scientists and microbiologists, as well as experienced technologists conducting the best-adapted diagnostic procedures for a tertiary-care center. All of this was dedicated to the identification of viral infections. At the end of this visit, I asked my colleagues “. . . but where are your viruses?” For few seconds, I had no answer, just silence. . .

I have accepted the task of defending viral culture and convincing the reader, or the jury, of its utility. Let me first make it clear that in 2012 the average clinical microbiology laboratory (where bacteriology, virology, mycology, and parasitology tests are performed under the same roof) does not need to grow viruses. For most of these laboratories, appropriate virological tests can be conducted without viral culture. In contrast, I believe that clinical laboratories specialized in virology; particularly if they are end institutions that care for highly immunocompromised patients, have to maintain their capacity to perform viral culture. A jury needs to make its final decision beyond any reasonable doubt. Defense lawyers are paid to defend their clients despite adverse evidence; their task is to ignite doubt in a juror's mind. There is much evidence against viral culture, but before we definitely kill off this procedure, the task of any microbiologist is to ask if this is justified beyond any reasonable doubt.

Recent advances in molecular technology have defied predictions, and powerful genome analyses have led to breathtaking scientific advances. New technologies for virus identification, ranging from next-generation sequencing to electrospray ionization mass spectrometry, are not yet routine but could be in a few years. In 2012, PCR-based assays and serological tests are the indisputable cornerstones of clinical laboratories for the identification and treatment of most acute or chronic viral infections. Compared to PCR, it is obvious that culture is less sensitive and the spectrum of cultivable viruses is largely restricted. Culture is clearly useless for the so-called “noncultivable” viruses. Group C rhinovirus, one of the most frequent causes of human infections, is a good example of an infection that has been missed for decades. The generic aspect of culture is an advantage that is part of daily life in bacteriology but does not exist in diagnostic virology; each virus requires predefined cell lines that immediately limit the breadth of detection. The use of mixed cell lines (1) could improve and facilitate the detection of multiple different viral genera or species, but the gain

is limited. Viral culture also needs serious expertise and astute technologists; all cell lines are not equal, and the ability of a given virus to grow is, in part, unpredictable. Those of us involved in influenza virus surveillance know that circulating human influenza viruses easily grow to high titers during a given season and become difficult to grow the year after. I could stop here and ask for a plea agreement and leave culture where it was in the 20th century. . .

Let us start with the potential utility of whole viruses to validate real-time reverse transcription (RT)-PCR assays. It is common practice, including in our laboratory, to use plasmids for PCR validation or for quantification assays, which results in wonderful amplification curves and where even one copy of a plasmid can be detected in a reaction mixture. However, this type of validation does not tell the whole truth and only indicates that the primers and probes used for the PCR are adapted to the cleanest template in the most favorable analytical situation. This is a serious limitation, since both the extraction and the retrotranscription are not controlled. Standardized viral stocks spiked into clinical specimens provide the unique opportunity to compare the analytical performances of different extraction procedures (2, 3) and reproduce as closely as possible original specimens. In the case of RNA virus, this also assesses the efficiency of RT and PCR detection in one complete procedure and experiment. Whole viruses also have the advantage of presenting RNA in its original configuration, including secondary structures that could impact the efficiency of retrotranscription. Endpoint dilutions can then help to compare the potential sensitivity of your assay with that of culture. For highly variable RNA viruses, such as picornaviruses or influenza A virus, the performance of molecular tests will vary according to the serotype, subtype, or genotype. The use of a library of viruses of different serotypes to validate a real-time RT-PCR targeting human rhinoviruses immediately highlights the relative performance and limits of a given assay according to the serotype (4). Thanks to culture, laboratories can create a large amount of their own extraction controls that are missing from many commercialized procedures.

Influenza diagnosis and surveillance are probably among the best examples of where culture still has a significant role. Each day and each season around the globe, influenza viruses are drifting. The pace and the pattern of this variability are unpredictable, even in the so-called “conserved” genes and regions. Primers and probes, particularly those used to subtype the virus, need to be updated on a regular basis (5, 6). Therefore, during the influenza season, viral culture remains part of the methods used to identify potential variants or animal viruses not detected by PCR. In 2012, antigenic characterization of influenza virus, vaccine selection, and assessment of the immune response are all dependent on viral culture. Hemagglutination inhibition and microneutralization assays are still the reference methods used to characterize the phenotypic and antigenic status of a given strain; this cannot be done by sequence analysis alone. Similarly, genotypic signatures of antiviral resistance are inferred from phenotypic and enzymatic assays that are culture dependent. The recent H1N1 virus outbreaks illustrate these issues well: the virus cross-reacted only partially or weakly with previous human viruses, and this was key information used early in the pandemics to devise new public health strategies. Although this goes beyond the average routine diagnosis, it is part of the duty of each clinical virology laboratory to participate in the isolation of new influenza virus variants. This should, of course, be done in adapted biosecurity facilities. It is also expected that both animals and reassortant influenza viruses could be easily missed by PCR assays targeting one given gene. When such viruses are

expected, culture is still a complementary method. Figures 1 and 2 describe a recent case of swine influenza detected in our laboratory that was rapidly confirmed by culture and not by the usual screening RT-PCR assay. This RT-PCR assay was revealed to be inefficient and provided a nonspecific signal that was initially considered a negative result. Sequence analysis directly from respiratory specimens also faces serious limitations, and growing the virus facilitates all sequencing procedures. In line with these observations, the ongoing investigation of the influenza A (H3N2) variant virus outbreaks needs culture to characterize the virus (<http://www.cdc.gov/flu/swineflu/h3n2v-outbreak.htm>).

Enterovirus 71 and the currently ongoing outbreak in Asia are associated with an unusual number of cases and severe complications (http://www.wpro.who.int/topics/hand_foot_mouth/en/), which seem to be caused by an uncommon re-emerging C4 genotype. At the diagnostic level, the virus can be detected in stool or respiratory specimens by RT-PCR. However, most assays target the conserved 5' noncoding region and do not have the ability to differentiate enterovirus 71 from other enteroviruses (e.g., coxsackievirus). A specific assay for a given enterovirus generally targets the capsid region (VPI), which is highly variable and subject to immune pressure. For this reason, PCR could miss new variants. During such outbreaks, stool culture remains a useful procedure with relatively high sensitivity, and a comparison with RT-PCR will rapidly confirm whether the molecular assay remains valid. In addition, virus isolation by cell culture provides an adequate quantity of virus for rapid and efficient whole-genome sequencing, as well as for phenotypic assessment. The neutralization assay is a reference test still used to assess the immune response and can be done only by using circulating strains provided by routine laboratories; the situation is similar for poliovirus surveillance (7). In addition, in resource-constrained countries, given the cost of molecular procedures, culture remains a key component of poliovirus surveillance, including vaccine-derived strains. Measles is also another disease where culture facilitates surveillance.

Clinical investigations which include culture have added significant information and have helped us to better understand the duration of RNA shedding and rhinovirus-related respiratory symptoms (8). The comparison of viral shedding and the duration of positive RNA detection is key information used to estimate the risk of transmission, to implement appropriate infection control measures, and to better assess the window of opportunity for treatment intervention. Studies comparing RT-PCR and culture for respiratory syncytial virus were useful in addressing the above-mentioned questions (9).

For latent DNA viruses, such as cytomegalovirus, PCR detection is very sensitive and the viral load in blood is used to evaluate the likelihood of associated disease. PCR is also used more and more frequently to test different biological fluids, e.g., bronchoalveolar lavage fluid collected from transplant recipients during a bronchoscopic procedure. Nevertheless, phenotypic methods for assessing herpesvirus resistance remain essential; relevant genotypic changes can be assessed only by culture-based methods. In addition, these culture-based procedures can indicate whether increasing drug levels can overcome resistance (10).

Beyond any routine diagnostic procedure, culture still has an important place in development and research. HIV is one of many examples. When developing the next generation of anti-HIV-1 drugs, one of the critical questions to address is the genetic barrier to resistance of new antiviral agents. Specifically, how difficult is it for the virus to develop mutations that confer reduced susceptibility to new anti-HIV-1 agents? *In vitro* resistance selection ex-

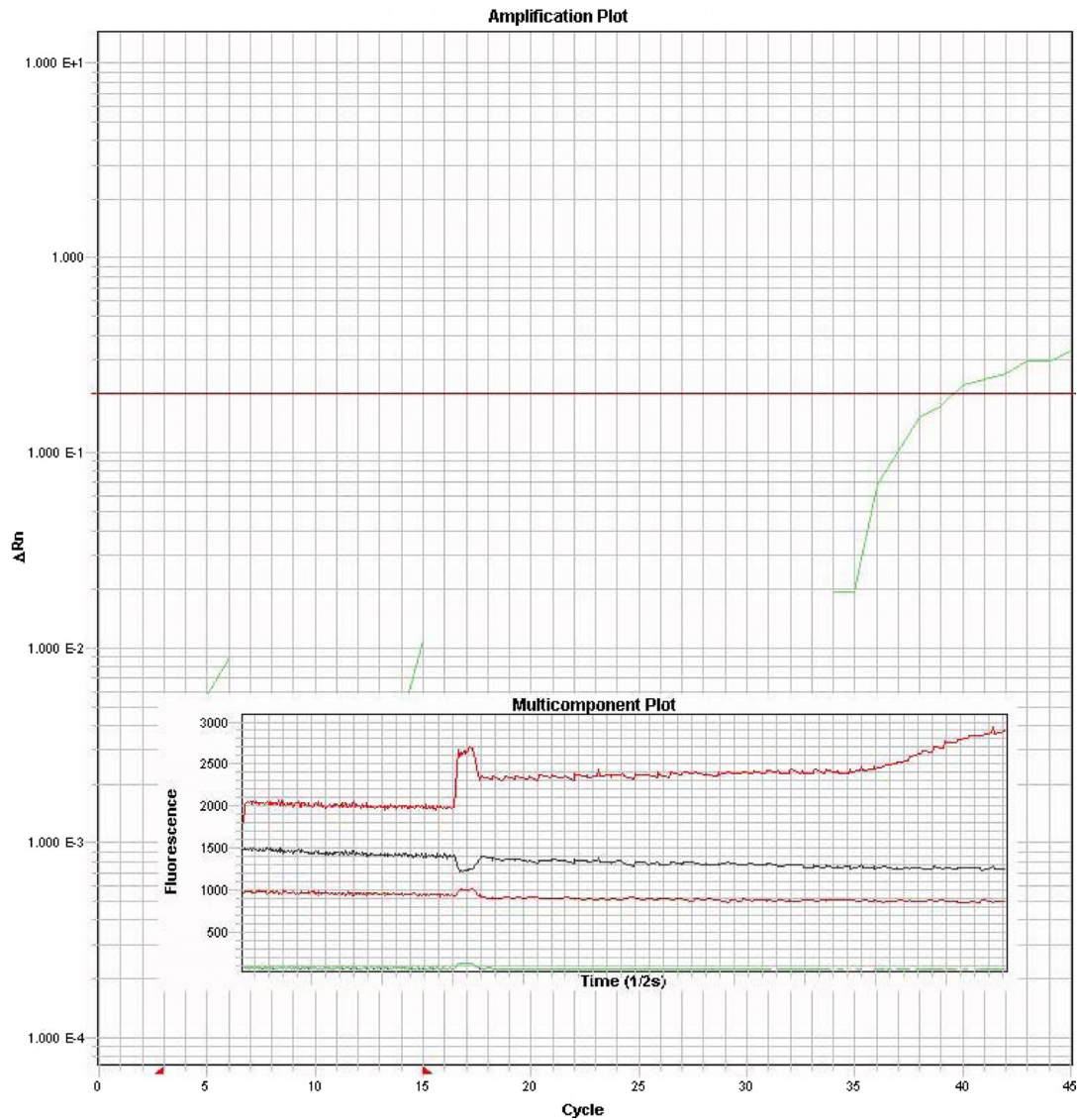


FIG 1 Identification of influenza A (H1N1) virus of porcine origin in a pig farm employee by RT-PCR versus culture (see Fig. 2). A nasopharyngeal specimen from a farm employee with an influenza-like illness was screened for influenza virus by using a panel of specific RT-PCR assays. A generic RT-PCR used to screen animal and human matrix gene sequences of influenza A viruses (5) provided a signal (shown are the amplification and multicomponent plots) considered to represent ambiguous or even negative results, and all other combinations targeting the hemagglutinin genes of human viruses (seasonal H1, H1 2009, and H3) (5, 6) remained negative (data not shown). ΔRn , delta of the normalized reporter value.

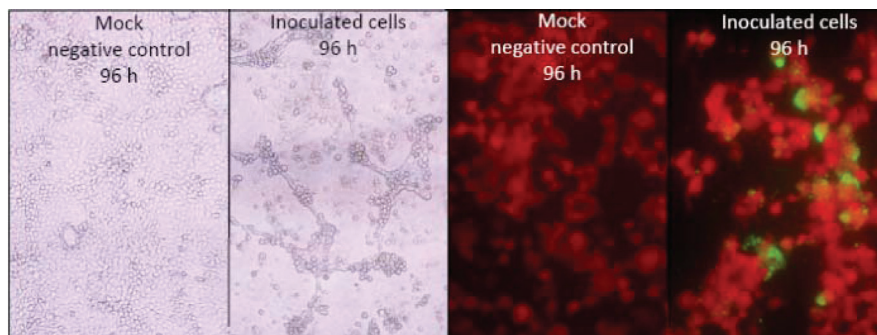


FIG 2 (Left) In contrast to what was observed by RT-PCR, influenza A virus was easily cultivated on SIAT-1 MDCK cells, and a strong and specific cytopathic effect could be observed after 96 h. (Right) Immunofluorescence analysis using monoclonal antibodies directed against influenza virus nucleoprotein confirmed the presence of viral antigens in cells. The final hemagglutinin sequence obtained from the cell supernatant and subsequent phylogenetic analysis revealed a virus similar to avian-like swine influenza viruses, which predominate in European pigs (GenBank accession no. [CY100132](#)). One mismatch, which was probably the cause of the ambiguous amplification, was observed in the reverse primer.

periments are still used to assess the genetic barrier of antiviral agents and to predict the potential resistance mutations that may emerge in clinical trials (11). Hepatitis C virus is another example where the lack of a virus culture system has baffled researchers.

It is obvious that in many situations, such as acute meningoencephalitis, systematic viral culture (in this case, of cerebrospinal fluid) is useless (12). For investigations of special cases in which a viral infection is suspected (e.g., hematopoietic stem cell transplantation, infants with primary immunodeficiency with persisting symptoms or fever), culture remains a useful complementary tool. However, by definition, the design of any real-time PCR assay is based on available viral sequences; the spectrum is thus limited, and variants can easily be missed. For virus discovery, new technologies, such as high-throughput pyrosequencing, have bypassed these limitations (13), but these technologies cannot be applied systematically, have a limited sensitivity, and could still benefit from viral isolation (14). A novel bunyavirus has recently been identified in China after the inoculation of clinical samples onto multiple cell lines; this virus has caused hundreds of cases of severe human diseases (15). Viruses such as metapneumovirus and coronavirus NL63 were all discovered as a result of culture. Another issue is related to the very large number of common or uncommon viruses that could cause infections in highly immunocompromised patients; in this population, viruses that generally cause self-limited diseases can cause unexpected opportunistic diseases. In the frequent clinical situation of prolonged fever and persisting unusual symptoms, the number of agents potentially involved is endless and includes viruses that are not systematically screened for. Performance of viral culture in selected cases is a complementary strategy that reveals useful information and needs to be maintained (16, 17).

Viral culture is not appropriate for routine daily results, but specialized laboratories should rely on their own ability to use viruses as controls, perform complete investigations when needed, and store representative clinical strains whenever possible. By maintaining their ability to conduct viral culture, these laboratories could also provide strains for future vaccine development and human isolates for research purposes. In the 21st century, who will still collect these clinical strains? Research laboratories use only very specific and adapted viruses that are often not appropriate for vaccine or antiviral development. Beyond their duty to provide clinicians with appropriate sensitive and specific tests in a timely manner, specialized clinical virology laboratories have additional responsibilities and tasks. One of these is to keep viral culture alive and use it when appropriate for specific investigations, surveillance, and quality control. This is not a decision based on the promotion of efficient or cost-effective procedures but rather a strategic decision for the future of clinical virology.

Laurent Kaiser

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SUMMARY

Points of agreement:

- Molecular testing has usurped viral culture for the routine detection of commonly encountered viruses.

- Viral culture is less sensitive than PCR and is more difficult and time-consuming to perform.
- Quantitation of viruses has been a major advance in monitoring of the response to therapy for HIV and hepatitis B and C virus infections.
- Viral cultures still have a place in diagnostic virology for the detection of new viruses or variants of well-recognized viruses that may be missed by molecular methods.
- Having individuals who are skilled in viral culture is important for antiviral drug and vaccine development, detection of new viral agents, and detection of drug-resistant viruses.

Issues to be resolved:

- Although it is clear that viral culture is still needed for a variety of reasons, it is unclear if this capability is truly required in a tertiary-care setting or if regional or national reference laboratories that can monitor the emergence of novel agents by culture are all that is needed.
- Training of individuals who can maintain competence in viral culture is a significant challenge for the future, as the numbers of individuals with these skills is declining and clinical laboratory science training programs offer little or no training in this skill.
- With the emergence of new viral strains that are not detectable by currently available molecular methods, it is unclear how nimble diagnostic manufacturers will be or what the impact of regulatory agencies on designing and bringing to market new diagnostics for emerging viruses will be.
- The recognition of multiple viruses in a single respiratory specimen is a clinical challenge currently with no clear understanding of the clinical significance of such a finding. This may also be true when molecular methods are widely available for detecting viruses in the gastrointestinal tract.

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