Published in final edited form as:

Vaccine. 2014 May 19; 32(24): 2916–2926. doi:10.1016/j.vaccine.2014.02.021.

Systematic Evaluation of *In Vitro* and *In Vivo* Adventitious Virus Assays for the Detection of Viral Contamination of Cell Banks and Biological Products¹

James Gombold^a, Stephen Karakasidis^a, Paula Niksa^a, John Podczasy^a, Kitti Neumann^a, James Richardson^b, Nandini Sane^b, Renita Johnson-Leva^b, Valerie Randolph^c, Jerald Sadoff^d, Phillip Minor^e, Alexander Schmidt^f, Paul Duncan^g, and Rebecca L. Sheets^{h,i}

^aCharles River Laboratories, 358 Technology Drive, Malvern, PA, 19355 ^bAdvanced BioScience Laboratories, 9800 Medical Center Dr. Bldg. D, Rockville, MD 20850 ^cFormerly with Wyeth, 401 N Middletown Rd., Pearl River, NY 10965 ^dCrucell, Newtonweg 1, 2333 CP Leiden, PO Box 2048, 2301 CA Leiden, The Netherlands ^eNational Institute for Biologics Standards and Control, Blanche Lane, South Mimms, Potters Bar, Hertfordshire EN6 3QG ^fGSK Vaccines, Rue de l'Institut 89, 1330 Rixensart, Belgium (formerly NIH/NIAID) ^gMerck and Co., Inc., WP17-101, 770 Sumneytown Pike, P.O. Box 4, West Point, PA 19486 ^hNIH/NIAID Division of AIDS, 6700B Rockledge Dr., Rm. 5145, Bethesda, MD, 20892

Abstract

Viral vaccines and the cell substrates used to manufacture them are subjected to tests for adventitious agents, including viruses, which might contaminant them. Some of the compendial methods (*in vivo* and *in vitro* in cell culture) were established in the mid-20th century. These methods have not been subjected to current assay validation, as new methods would need to be. This study was undertaken to provide insight into the breadth (selectivity) and sensitivity (limit of detection) of the routine methods, two such validation parameters. Sixteen viral stocks were prepared and characterized. These stocks were tested in serial dilutions by the routine methods to establish which viruses were detected by which methods and above what limit of detection. Sixteen out of sixteen viruses were detected *in vitro*, though one (bovine viral diarrhea virus) required special conditions to detect and another (rubella virus) was detected with low sensitivity. Many were detected at levels below 1 TCID₅₀ or PFU (titers were established on the production cell line in most cases). In contrast, *in vivo*, only 6/11 viruses were detected, and 4 of these were detected only at amounts one or more logs above 1 TCID₅₀ or PFU. Only influenza virus and vesicular stomatitis virus were detected at lower amounts *in vivo* than *in vitro*. Given the call to reduce, refine, or replace (3 R's) the use of animals in product safety testing and the emergence of

¹The views expressed are solely those of the authors and do not represent the National Institutes of Health, federal government policies, or company positions.

ⁱCorresponding author: CAPT Rebecca L. Sheets, Ph.D., USPHS, NIH/NIAID Division of AIDS, 6700B Rockledge Dr., Rm. 5145, Bethesda, MD, 20892.

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new technologies for the detection of viruses, a re-examination of the current adventitious virus testing strategies seems warranted. Suggested pathways forward are offered.

Keywords

Vaccines; Viral Safety; *In Vitro*; *In Vivo*; Adventitious Agents; 3Rs

INTRODUCTION

Adventitious agent tests are routinely used to assess safety and purity of cell banks and biologics. The methods used have ensured that very few products have reached the market with viral contaminants, and in the cases where they have, the contaminants have not posed a risk to human health. The *in vivo* and *in vitro* assays currently in use were developed more than 50 years ago based on clinical diagnostics and originally were used to detect specific adventitious agents known to be possible contaminants in vaccines[1]. Subsequently, these assays became widely used as broad general screening assays for known and unknown potential contaminants.

Generic tests for viral contaminants of biologics include electron microscopy, assays for retroviral reverse transcriptase, and detection of virus in cell culture and animal host systems. Virus detection *in vitro* has been accomplished using multiple cell lines to which sample is applied and subsequent observation for cytopathic effect (CPE), hemagglutination (HA), or hemadsorption (HAD). *In vivo* assays involve inoculation of specific animal species and subsequent observations for mortality and testing of tissue for the presence of hemagglutinins. These assays have been considered necessary to detect new or emerging viruses and to complement narrowly focused virus-specific assays such as PCR that came into routine use later.

The use of multiple and distinct cell lines and animal hosts stems from the knowledge that viruses can have a limited host range (i.e., infect certain species but not others), or exhibit tropisms for specific cell types or tissues (e.g., grow in kidney epithelial cells but not fibroblasts). Certain hosts systems may be more permissive for virus replication and therefore more likely to show CPE *in vitro* or mortality *in vivo*. Animal hosts were included to detect agents that are difficult to propagate *in vitro* or are non-cytopathic or cause immune-mediated disease, e.g., lymphocytic choriomeningitis virus.

In 2004, The Vaccine Cell Substrates conference focused, in part, on the scientific issues related to cell substrates used for vaccine manufacture. A major theme of the conference was adventitious agent testing, with discussions on the validity of existing tests as well as discussions of new tests being developed. The goal of these discussions was to develop consensus recommendations on issues regarding these tests and to identify research gaps that have precluded informed decision-making. A summary report of the meeting was published in [2]. This meeting produced the following consensus recommendations:

1. Regulatory requirements and methods should be harmonized amongst regulatory bodies/agencies.

2. Viral safety assurance stems from compliance with good manufacturing practices (GMP), manufacturing consistency, and adequate quality control, including partial redundancy of the previously relatively uncharacterized tests commonly used.
3. The *in vivo* tests continued to add value and could not be eliminated at that time.
4. New methods for characterizing cell substrates should continue to be developed. It may be easier to use new tests with new products than to replace tests for products already licensed.

The Workshop on Microbial Agents in Animal Cell Substrates: Update on Testing and Methods held April 20-21, 2009, reinforced the findings of the earlier conference in 2004. In particular, the newer methods were coming closer to being introduced into routine testing or cell bank characterization. Despite a considerable number of *in vivo* tests having been performed over the past many years, it was reported at this conference that no adventitious agents were detected in this way that were not also detected using *in vitro* methods. While there remained reluctance to eliminate animal-based testing, there was recognition that given the “3 R's” policy to reduce, refine, or replace the use of animals in product safety testing, justification for use of the *in vivo* methods needs continued consideration.

Although there is some information available, the breadth and sensitivity of these assays have not been assessed systematically and publicly reported. With respect to the *in vitro* and *in vivo* adventitious agent tests, the Vaccine Cell Substrates 2004 meeting participants concluded that the sensitivity and breadth of existing tests are presumed from historical experience and should be evaluated systematically. The data obtained from this research would then be available to use as a baseline for comparison with newly emerging tests and for consideration of implementing the 3 R's with respect to *in vivo* testing.

METHODS

Project Design

An expert panel, which included international regulators, vaccine industry, and NIAID intramural and extramural staff, was convened to design and guide the project. Their input determined the scope of the project, as well as practical aspects. Although the project was not intended to support a particular regulatory submission, it was intended to aid manufacturers and regulators in decision-making about test utility, capabilities, and implementation of the 3 R's. Therefore, it was agreed that the tests should be performed in accordance with Good Laboratory Practices (GLP), in order to ensure the quality, credibility, and integrity of the data. Furthermore, the list of viruses to be considered and which test systems to apply for each virus were determined by this panel.

Cells and Viruses

Cells used for this study were obtained from cell banks prepared and used for routine testing by Charles River Laboratories (CRL). Eight cell lines were used to prepare 16 Research Virus Stocks (RVS; Table 1). Cells were grown at 37 °C in EMEM supplemented with either 5% or 10% irradiated fetal bovine serum (FBS, SAFC Biosciences).

Seed viruses were obtained from stocks available at CRL or purchased from Zeptomatrix (Franklin, MA). The seed viruses and RVS were stored at -70°C . Virus stocks were tested post-manufacture for the presence of contaminating bacteria and mycoplasma and titrated by either plaque assay, 50 percent tissue culture infectious dose (TCID₅₀) assay, or by immunofluorescence (FAID₅₀). The cell line used for propagation of the virus was used for titration, except for HSV, SV40 and VSV, which were grown in Vero cells but assayed using Vero 76 cells, as well as rubella virus, which was grown in BS-C-1 cells but assayed using RK-13 cells.

The identity of each RVS was generally determined in immunofluorescence assays (IF) using virus-specific antisera. The only exception was rhinovirus 2, where sequencing of reverse-transcribed genomic RNA was used to establish identity.

Additional details for the propagation and characterization of the viruses will be available for distribution with the virus stocks. The virus stocks will be made available to researchers developing new assay methods for the purpose of adventitious agent testing through the DAIDS Reagent Resource Support Program for AIDS Vaccine Development.

Animals and Embryonated Hens' Eggs

Specific Pathogen Free (SPF) animals and eggs used in this study were obtained from CRL facilities accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International, and all experimental work on live animals was approved by the CRL Institutional Animal Care and Use Committee. Animals were housed in filter-topped Microisolator® cages in a negative-pressure work area (100% exhausted), fed *ad libitum* with irradiated feed (Lab Diet 5L79; Purina Mills, Richmond, IN) and sterile filtered water access, and were maintained on a 12 hour light-dark cycle. All SPF colonies and flocks were shown to be free of adventitious infections.

In Vitro Adventitious Virus Assays

In vitro testing for adventitious agents was done in compliance with the 2010 U.S. Food and Drug Administration (FDA) Guidance for Industry [3], European Pharmacopoeia 2.6.16 [4], and ICH Q5D [5]. To assess the ability of standard adventitious virus assays to detect potential viral contaminants, serial 10-fold dilutions of each RVS were inoculated onto MRC-5, HeLa, and Vero cells grown in 6-well plates. A fourth cell line was added for six viruses (adenovirus 5, adenovirus 41, bovine viral diarrhea virus, Coxsackie A16 virus, simian cytomegalovirus, and rubella virus, Table 2). In those cases, the production cell line was used to ensure that at least one cell line was capable of propagating the virus, to serve as a positive control in the assays. In general, each RVS was diluted in growth medium to achieve a range of 100 to 0.0001 infectious units (PFU, TCID₅₀, or FIAD₅₀) per well as defined by titration on the production cell line; one plate (six wells) was inoculated with each dilution. In cases where the highest dose (i.e., 100 infectious units) was not detectable on a given cell line, additional assays were done using virus titers of up to 1×10^6 infectious units to determine a detectable amount, up to that titer.

Following adsorption for 60 minutes, the cells were fed with EMEM containing FBS and incubated at $36.5 \pm 1^\circ\text{C}$ in 5% CO_2 . Monolayers were observed for CPE at regular intervals for 14 days. Growth medium was replenished as required. On day 14, cells were removed from the incubator and tested for hemagglutination (HA) or hemadsorption (HAD) of red blood cells (RBCs) (excluding those tested with rhinovirus 2, VSV, and BVDV). Briefly, in cases where HA was assessed, an aliquot of growth medium was removed and tested for hemagglutination by incubation with a suspension of chicken, guinea pig, or human erythrocytes following incubation at 4°C , room temperature, and 36.5°C . For measles virus, monkey erythrocytes were substituted for human erythrocytes. In cases where HAD was assessed, cell monolayers were washed, and duplicate wells were overlaid with a suspension of chicken, guinea pig, or human erythrocytes. As with the HA testing, for measles virus, monkey erythrocytes were substituted for human erythrocytes. The plates were observed for adsorption of the RBCs to the cell monolayers after sequential 30 minute incubations at 4°C , room temperature, and 36.5°C . Three replicate assays were performed.

A blind passage of the growth medium from cells was done for all RVS samples that appeared to be morphologically normal, i.e., without CPE, on day 14. These monolayers were observed for CPE at regular intervals for an additional 14 days. Growth medium was replenished as required. On day 28, cells were removed from the incubator and tested for HA or HAD of RBCs as described above. In addition, cells that had been exposed to BVDV were collected and stained for the presence of non-cytopathic BVDV by IF using BVDV-specific antiserum (National Veterinary Services Laboratories, Ames, IA).

The Limit of Detection (LOD) for each virus on each indicator cell line (MRC-5, Vero, and HeLa) was defined as the lowest virus inoculum that scored positive in all three assay runs by any of the endpoints used.

***In Vivo* Adventitious Virus Assays**

In vivo testing was conducted to comply with 21CFR Part 630.18 [6], 21CFR Part 630.35 [7] (revoked after 1996), 21 CFR Part 630.55 [8], 21 CFR Part 630.65 [9] (revoked after 1996) on which current adventitious agent testing for vaccines is based in the U.S., as well as with current 21CFR Part 58 Good Laboratory Practices [10]. *In vivo* assays were performed using suckling mice, post-weaning mice, and embryonated hens' eggs. Details of the host systems, including ages, weights, inoculation routes, and inoculation volumes are shown in Table 3.

The *in vivo* experimental design was biphasic, testing first for breadth of detection using undiluted virus and then testing serial dilutions to establish sensitivity. In the first experiment, mice or eggs were inoculated with undiluted RVS and observed for mouse mortality or death of the embryo. If mortality was below 20% in chicken eggs or suckling mice, specimens from surviving embryos or suckling mice were passaged into naïve embryonated eggs or suckling mice according to the standard "inapparent virus" protocol. (This test is so-called, as it was intended to be able to detect viruses that might be inapparent in cell culture infection, as SV40 was in rhesus cells when it was first discovered.) For RVS that caused mortality exceeding 20% in the initial testing, a limit of detection (LOD) of the assay was determined by inoculating mice or eggs with serial 10-fold dilutions of the RVS.

In order to minimize cross-contamination, only one virus was evaluated in the laboratory at a time. In addition, open manipulations of virus were performed in a Class II Biological Safety Cabinet, and animals were housed in filter-topped Microisolator® cages in a negative pressure work area (100% exhausted).

Unlike the *in vitro* tests, which were run in replicate assays, the *in vivo* methods were run one time at each dilution, if dose-response sensitivity testing was warranted by the outcome of the breadth analyses. This was done to minimize animal use. Therefore, a “reliable” LOD for the *in vivo* test could not be established, for the sake of comparison with what was done for the *in vitro* methods. LOD was defined by the lowest inoculum that resulted in 20% mortality or evidence of viral infection. The titers of the viral stocks were established in cell culture as indicated in the methods section, so the LOD *in vivo* could be lower than 1 if the *in vivo* test systems were more sensitive. The compendial method defines a failing test as one in which more than 20% of the animals die on test or if any animals show signs of infection by an adventitious agent. Setting the threshold at 20% mortality is a practical compromise, since it is not unusual to lose animals or eggs to injection trauma, cannibalization, or other microbial contamination relating to the injections. So, the lowest inoculum at which 20% or more of the animals died was considered the LOD for that virus/*in vivo* test system pairing.

RESULTS

Detection of Adventitious Viral Agents by *In Vitro* Assays

The purpose of this study was to systematically assess the sensitivity of commonly used cell lines to detect infection by a number of different viruses, representing those that might contaminate vaccines in production adventitiously from the production environment (e.g., cross-contaminating vaccine strains), personnel (e.g., respiratory viruses), culture media (e.g., bovine viruses), or production cell substrate (e.g., certain simian or human viruses). The research virus stocks prepared during the initial phase of this project were diluted and inoculated onto MRC-5, HeLa, and Vero cells. As shown in Table 2, a fourth cell line, representing a positive control cell line, was used in studies of adenovirus 5, adenovirus 41, BVDV, Coxsackie A16 virus, rubella virus, and sCMV, to ensure that at least one cell line would score positive. Cells were monitored for CPE for 14 days, after which the conditioned growth medium was used to inoculate new monolayers of the same cell lines if CPE had not yet been observed. Cells were monitored for an additional 14 days. Hemadsorption (HAD), hemagglutination (HA) or immunofluorescence (IF) assays were done on days 14 and 28.

The limit of detection (LOD) for each virus and cell line pair was defined as the lowest amount of virus that was detectable in all 3/3 repeat assays by CPE, HA, HAD, IF, or any combination of those endpoints. A given dilution in a given run was judged positive if at least one of the six wells showed evidence of viral infection. In many cases, one or two replicates may have been positive at higher dilutions, but if not reliably detected (3/3), then these higher dilutions were not considered to be the LOD because one could not be assured that dilution would always be detected. Therefore, the LOD determined in this manner might not be the true LOD, but higher than the true LOD. It should also be noted that the titer was

frequently performed on the production cell line, and other cell lines may be more sensitive (e.g., detecting less than 1 infectious unit as determined on the titration cell line).

All viruses that were tested had an LOD of at least 1 TCID₅₀ on at least one cell line, but in some cases an LOD below 1 TCID₅₀ was observed (Table 4 and Figure 1). The most likely reason that LODs below 1 TCID₅₀ were observed was that the adventitious virus assays were conducted over 28 days rather than the much shorter time used for titration of the research virus stocks (approximately seven days or less). The longer assay duration, combined with the passage of conditioned media onto new indicator cells, would markedly increase the sensitivity of the adventitious virus assay. Additionally, differences in the susceptibility of the various cell lines to infection may explain low LODs for three of the viruses (Ad41, CoxB, and Echo 11). In each of these cases, the viruses were propagated and titered on a cell line other than MRC-5, Vero, or HeLa. For example, Ad41 was titered on A549 cells, but showed an LOD of 0.1 TCID₅₀ on HeLa cells. Similarly, Echo 11 was titered on LLC-MK2 cells but had an LOD of 0.01 TCID₅₀ on both MRC-5 and Vero cells.

The three standard indicator cell lines (MRC-5, HeLa, and Vero) were able to detect the presence of the test viruses at levels 0.1 infectious units per 0.5 mL inoculum or less for 10 of the 16 viruses (Table 4). The LOD for SV40 was slightly higher at 1.0 infectious units per 0.5 mL inoculum. Influenza A and rubella did not cause significant CPE in the standard indicator cell lines. Influenza was readily detected by HAD on MRC-5 cells, but not on HeLa or Vero cells. Rubella was poorly or not detectable by HAD on all three standard indicator cell lines, but caused CPE and positive HAD reactions at low (0.1 TCID₅₀ per 0.5 mL) levels on RK-13 cells. As expected, non-cytopathic BVDV was not detectable on the MRC-5, HeLa, and Vero cells, by CPE or IFA, but was detected by IFA on the production cell line (BT).

None of the viruses required a 28-day assay for detection provided that appropriate cell lines were used, although significant increases in sensitivity were observed by day 28 for Ad41 and sCMV (see Supplemental Data, Table S1). For example, on day 14, Ad41 was detectable only at high titer (1.0×10^5 TCID₅₀) on the standard cell lines. Inclusion of A549 cells in the assay allowed detection of 10 TCID₅₀. However, extending the duration of the assay to 28 days afforded similar sensitivity on both MRC-5 and HeLa cells.

The strain of BVDV used in this study is non-cytopathic and was not expected to be detected by CPE. IF staining of infected cells is used to detect BVDV, and when done on BT cells, the LOD at day 28 was 0.1 FAID₅₀ (not shown). The limit of detection for BVDV on day 14 was not determined.

HAD and HA was done using chicken, guinea pig, and human erythrocytes (note that for measles virus, monkey erythrocytes were used instead of human erythrocytes; see Supplemental Data, Tables S2 to S5). In general, virus-infected MRC-5 and Vero cells were most likely to show hemadsorption for those viruses known to bind red blood cells. HAD was observed less consistently with mumps-infected MRC-5 cells compared to Vero and HeLa cells. Rubella was poor at inducing HA regardless of cell type but detectable on RK-13, MRC-5, and HeLa cells. Overall, HAD was best observed at 14 days post-infection

due to the destruction of cell monolayers at the higher inoculum titers by day 28 (e.g., CAV, CBV, Echo, HSV, and sCMV on MRC-5 cells; Ad41, CAV, CBV, Echo, HSV, and influenza virus on Vero cells; and CAV, CBV, Echo, and SV40 on HeLa cells).

Detection of Adventitious Viral Agents by *In Vivo* Assays

Inoculation with Undiluted Virus Stocks—Mice and/or embryonated hens' eggs were inoculated with undiluted RVS as described in Table 3. For six of the viruses, mortality exceeded 20% in one or more of the given test systems (Table 5). Five of the viruses tested were not detected *in vivo* even at the undiluted concentration.

Measles virus, BPIV-3 and rubella virus did not cause significant mortality of chicken embryos after primary inoculation or passage; mumps was lethal in eggs by at least one route (Yolk Sac). In mice, BVDV or echovirus did not cause significant mortality. VSV was the only virus tested in all host systems and was lethal to all of them. Significant mortality was caused by undiluted stocks of HSV and the Coxsackie viruses in mice and by the undiluted stock of influenza virus in chicken embryos. It should be noted that blind subpassage performed in suckling mice and in eggs did not result in additional mortality of significance.

Table 6 shows the day post inoculation (DPI) on which mortality exceeded 20% when the *in vivo* system was inoculated with undiluted viral stocks, which ranged from 2 to 8 DPI, and the percent mortality on that day, which was 100% for 7 of 11 pathogenic virus-host combinations. Virus stocks for which greater than 20% mortality was observed without dilution were then studied in a titration protocol, to determine an LOD (Figure 1).

Inoculation with Diluted Virus Stocks—Based on the sensitivity protocol (titration protocol), stocks that scored positive at the undiluted concentration, were serially diluted and each dilution tested to establish an LOD or amount at which a particular viral stock would score positive with that amount or more and negative with less than that amount. Only two viruses (influenza virus and VSV) scored positive in eggs, or in all test systems, respectively, at titers lower than they were detected *in vitro*. The other four viruses that scored positive *in vivo* were only detected at titers higher than 1 TCID₅₀ or PFU as follows: Coxsackie A virus at 10⁵ TCID₅₀, Coxsackie B virus at 10 TCID₅₀, HSV at 100 PFU, and mumps virus only at the undiluted concentration.

Pathology noted in infected animals—There was no pathology information collected on animals assigned to the breadth study. Per protocol animals that died on study were submitted for necropsy, but only a gross exam was conducted; tissues were harvested to be used in subpassage, not for histological examination.

A total of 5 mice from each of the inoculation groups with high mortality rates assigned to the HSV sensitivity study were submitted for necropsy and subsequent histopathology. Because the majority of the animals were found dead, and not euthanized, all tissues were variably affected by autolysis. However, consistent lesions were identified in both post-weaning and suckling mice, indicating that the mortality seen on study was directly related to the test article. Both post-weaning and suckling mice were noted to have lesions in the

brain, while the livers of the suckling mice were also affected and were indicative of infection with HSV. Among the three post-weaning mice in the HSV study that were euthanized because of morbidity, the observed meningoencephalitis was noted as marked in severity, whereas those animals found dead had similar findings that were noted as moderate in severity. Similarly, lymphoid necrosis of the spleen that was observed in 2/3 post-weaning mice euthanized was noted as mild in severity, whereas in those found dead, the severity was minimal (as it was in the remaining post-weaning mouse that was euthanized).

Five animals from six inoculation groups of post-weaning mice and varying numbers of animals from four litters of suckling mice with high mortality rates assigned to the VSV sensitivity test were submitted for necropsy and histopathology. Autolysis of the tissues collected from suckling mice made evaluation of the samples difficult. Those that were able to be evaluated consistently showed periventricular and/or perivascular multifocal necrosis of the brain, considered by the pathologist to be the result of administration of the test article. Post-weaning mice all had perivascular and/or meningeal brain lesions; one mouse had a lesion that extended into the spinal column. Significant lesions were not noted in other tissue. Since all mice became ill and died after being injected with the test article (titrated VSV), and because all had similar microscopic lesions, causality seems established.

Necropsy was not performed on the Coxsackie A virus sensitivity test, as 4 mice were missing from the litter, presumably cannibalized. Because the cause of the mortality cannot be definitively linked to viral infection, the LOD could not be definitively determined by the sensitivity test.

The autolysis of the tissues from the suckling mice assigned to the Coxsackie B virus sensitivity test made evaluation difficult. While consistent liver lesions were noted, autolysis made the definitive determination of the necrosis difficult.

Comparison of sensitivity and breadth of *in vitro* and *in vivo* methods—For almost every virus studied the *in vitro* assay was more sensitive than the *in vivo* assay (Figure 1). In addition, the *in vitro* systems used for the routine test (MRC-5, Vero, HeLa) were able to detect the test viruses in all but one case, for BVDV, although rubella was detected with low sensitivity; whereas the *in vivo* systems were unable to detect 5/11 viruses tested.

DISCUSSION

When poliovirus vaccines were licensed in the 1950's and as measles, mumps, and rubella vaccines were licensed in the 1960's, manufacturers were required by the FDA and other regulators around the globe to apply tests for viral safety and product purity, i.e., to show freedom from contaminants like viruses [3,4,5,6]. These tests were required to be performed by inoculating samples of vaccine harvest, or viral seeds, and production control cells onto indicator cell cultures and into animals. Production control cells were required because these products were prepared in primary cell cultures, and some vaccines in current use continue to use similar cell substrates. These test methods, which were evaluated in the current study, persist to this day and are broadly applied for testing cell substrates and biological products.

They have largely been successful at preventing contaminated biologicals from reaching the market. In the few instances that virally contaminated vaccines have reached the market, the contaminants have not posed a risk to human health. In addition to the “routine” tests, manufacturers now also commonly utilize transmission electron microscopy and reverse transcriptase assays for detection of retroviruses, as well as molecular methods specific for known potential contaminants in order to assess viral safety in biologicals and the cell substrates used for their production. As new methods become available, more and more tests are added to the list of tests required or recommended by regulators. This never-ending addition to the list is not a sustainable or rational approach. However, regulators are reluctant to eliminate anything from the list without strong justification, particularly the tried-and-true. In light of the 3 R's policy to replace, refine, or reduce the use of animals in product safety testing and in the face of many impending new technologies, this project attempted to bring into the public domain data to support regulatory decision-making and policy development. These data are intended to provide manufacturers and regulators information about the relative capabilities of the routine tests. They also provide a baseline for developers of new methods to serve as comparators for what they might try to achieve or exceed with regards to sensitivity and breadth of detection.

When originally proposed for testing monovalent bulk harvest of live viral vaccines, the routine methods were used to test the greater of 500 doses or 50 mL (in the case of cell cultures) or 100 doses / 10 mL (in the case of eggs), and specified volumes and numbers of animals (in the case of mice)[4]. Currently however, lot sizes and culture volumes are much greater than they may have been when the tests were first promulgated (e.g., as large as 20,000 L bioreactors), and so the percentage of the harvest or lot being tested may be highly variable from product to product. Further, statistical sampling considerations are not generally applied. In fact, most testing is performed by contract service providers, and, although custom test protocols are available, they generally perform the same test for all samples. The volumes applied are those that have been validated for the standard test as run by that service provider, often as little as 1 mL/cell line. Similarly, the number of animals and inoculation volumes are invariant from product to product. It is important that a statistically valid sampling plan and a science-driven and rational testing strategy be implemented, as we begin to incorporate new methods into the standard testing. Although the tried-and-true have served the vaccine industry well over the years, assuring that safe and pure products are released to the market, there are some notable exceptions (e.g., SV40 found in poliovirus vaccines in 1960's[11], porcine circovirus found in a rotavirus vaccine in 2010[12]) that point to the need to reconsider our approaches to adventitious virus testing. Furthermore, since the aim of continuous improvement is implicit in Good Manufacturing Practices and in Quality by Design principles, we should also continue to improve our safety testing strategies. The advent of new technologies like deep sequencing, mass spectrometry following broad-spectrum PCR (Plex-ID), or microarrays, each with tremendous potential, albeit only capable of assessing very small sample volumes, also requires us to examine the list of tests and consider our course carefully moving forward.

In planning these studies, consideration was given to pragmatic concerns and, as a result, some compromises had to be made. Although a large number of viruses were included in the study, the viruses chosen do not represent every viral family. Some of the excluded viral

families are known not to be detectable by these tests (e.g., *Retroviridae*, *Papillomaviridae*, *Hepadnaviridae*) and special assays (retroviridae) or specific PCRs are used to detect members of those viral families. For practical reasons, the number of viruses had to be limited in this initial study. Also, because the program supporting the project focuses on vaccines, viruses that might be of greater concern for other types of biologicals, e.g., known contaminations that have occurred include Cache Valley virus [13] and reovirus 3 [14], were not included, although these contaminations were found by the *in vitro* cell culture assay when they occurred. Furthermore, the origins of the tests reflect their use in viral safety testing of vaccines, so the original purposes of the test methods were considered in the planning process. For example, the suckling mouse test was incorporated into vaccine safety testing specifically to detect potential Coxsackie virus contaminants and therefore Coxsackie viruses were included in the evaluations. Essentially, this study set out to test the null hypothesis that there was no qualitative or quantitative advantage of the *in vivo* tests over the *in vitro* tests, and allow the opportunity to reject the null hypothesis by finding significant examples in which the *in vivo* tests were compellingly more broad or sensitive and thus desirable to maintain in routine characterization of vaccine cell substrates. While the study was constrained by very practical limitations, the results are nonetheless enlightening.

Since propagation and banking of the viral stocks was necessary in order to characterize them prior to use in the routine tests, the viruses selected all grow in cell culture in some cell line. This approach could have influenced the relative assessments of growth *in vitro* vs. *in vivo*. Whether this accounts for some viruses expected to be detected reliably *in vivo* being detectable more sensitively *in vitro*, e.g., Coxsackie A virus, is unclear. Limited adaptation to growth in culture is not expected to eliminate the ability to grow *in vivo*, although the titer required to infect animals may be altered to some extent (e.g., by attenuation), but this limitation to the study must be recognized. It is also recognized that most adventitious agents appear to arise from the animal-derived culture components, i.e., bovine serum or porcine trypsin, and these contaminants would be expected to be wild-type viruses (i.e., unadapted). As will be discussed below, however, the adventitious contaminants of greatest concern would be those that can propagate in the production cell culture, and so the ability to grow in culture would be an expected characteristic. Further, there is no evidence to suggest the *in vivo* assays are more sensitive for wild-type viruses. For example, human influenza viruses, particularly H3N2 strains, can be difficult to propagate in eggs, until they are adapted. It should be noted that there can also be variation between different strains of the same virus with regards to their ability to propagate in cell culture, but due to practical limitations, only one strain of each virus was studied.

Other important facets not addressed in these studies would be the matrix effects of the test material and the differences in behaviors of viral variants: not only the difference between wild-type viruses and culture-adapted strains, as discussed above, but also between different culture-adapted variants (e.g., MVM) or between different wild-type strains (e.g., mumps viruses).

In the current era, new assays are required to be validated according to the principles outlined in the ICH guidance document[15]. However, compendial methods are required only to be verified for their intended use. Thus, these routine tests have not been subjected to

the more stringent assay validation principles. Although these studies did not attempt to fully validate all relevant performance parameters, two aspects were studied – the sensitivity in terms of a limit of detection for each method (cell line or *in vivo* test system) and each virus and the specificity in terms of breadth of viruses detected by each method. Also, even though the tests are intended to be broad spectrum, due to pragmatic limitations, only 16 model viruses could be studied. It should be noted that despite the long history of use of these routine tests, no international standards or reference standard materials exist for the purposes of standardizing the assays or for proficiency assessments.

An assumption that has been made to support the continued use of the *in vivo* methods has been that some viruses do not grow readily in cell culture and would be more sensitively detected by the *in vivo* test systems. These studies included viruses that were expected only to be detectable *in vivo* or to be more sensitively detected *in vivo*. These included the Coxsackie viruses and mumps virus, for which mice and eggs were considered most sensitive, respectively. Coxsackie viruses could be adventitiously introduced from the operators, environment, or starting isolate for the vaccine viral seed, and mumps virus, for instance, could result from cross-contamination in viral vaccines in facilities that also make mumps vaccines.

The results show that, in fact, all of the viral stocks, except BVDV, were detected by the *in vitro* test (using the combination of MRC-5, Vero, and HeLa as indicator cell lines). Non-cytopathic BVDV was detected only in the positive control cell line, BT (commonly used in the bovine virus test, though not the “routine” cell culture test) and only with the IFA endpoint (as expected, since a non-cytopathic strain was intentionally selected for study). Rubella virus was detected, but with uniquely poor sensitivity. Apart from these exceptions, the *in vitro* indicator cell cultures were reliably and highly sensitive. The cell lines may vary in this routine test, because the requirements are to include human diploid cells (which may include primary cell cultures, although often a non-diploid cell line is used), monkey kidney cells (could be primary cell cultures, but generally the Vero cell line is used), and a cell line of the same species and tissue type as the production cell line. Since these studies were undertaken generally and not to support a specific product, a decision was made to include the 3 most commonly used indicator cell lines for vaccines that might be propagated in a human or simian cell line (e.g., MRC-5, Vero). Although chick embryo fibroblasts (CEF) are usually included when propagating a vaccine virus in eggs or CEF, and Chinese Hamster Ovary (CHO) cells would be used when producing a biological from CHO cells, these cells were not included due to the pragmatic limitations of the study. For human or non-human primate cell lines used in production of vaccines, the 3 cell lines used most frequently in this test are MRC-5, HeLa, and Vero, and therefore we choose these cell lines to include for study. They proved to have a broad “specificity” or range of virus types that could be reliably detected at reasonably sensitive titers. Often amounts as low as 0.1 or 0.01 TCID₅₀ were detectable (see Figure 1 for details). It is important to remember that the titer values were in most cases determined on the production cell line for the particular RVS and the *in vitro* test is designed to be more sensitive than typical titration assays in that there is ample opportunity for amplification of input virus and the test is of much longer duration. So, dilutions below what was determined as a 50% endpoint titer on the production cell line

were able to be reliably detected on the indicator cell lines used in the tests in most cases (10 of 16 viruses tested, see Figure 1).

In fact, for several viruses, substantially less than 1 infectious unit (as defined by titration assays) was defined as the LOD in 6-well plates (Table 4). Notably, this happened even on detection cell lines that were the same as used to determine the titers. For example, an LOD of much less than 1 infectious unit was observed for Ad5 in A549 cells; for measles virus, mumps virus, BPIV-3 in Vero cells; for Rhinovirus 2 in HeLa cells; and for rubella virus in RK-13 cells. This suggests that the format of the detection assay was more sensitive than the format of the titration assay, in which there were several differences including the length of incubation and observation. Therefore, standardization of the format of the assay should be considered to control for potential differences in LOD due solely to assay format (e.g., while length of culture is specified, inoculum volumes, materials to be inoculated, size of culture, i.e., T-flask vs. 6-well plate, and other details are not specified and can vary by service provider). Development of international standards might also help to ensure reliable uniformity of the results from the *in vitro* methods.

Viruses that were expected to be detected in the *in vivo* test systems surprisingly were not detected in the test systems used or were only detected at such high titers that the *in vitro* methods were orders of magnitude more sensitive. Mumps virus was only detected in chicken eggs (yolk sac route) at an amount of 4.8×10^4 TCID₅₀. In contrast, mumps virus was detected in HeLa cells at 1 TCID₅₀ and in MRC-5 and Vero cells at 0.1 TCID₅₀ by HAD. Mumps vaccine is prepared in chicken embryo fibroblast cultures, but this virus did not propagate readily in chicken eggs inoculated *via* the routes used in these studies.

Historically, Coxsackie A viruses were expected only to be reliably detected in suckling mice, and both Coxsackie A and B viruses should be detectable in this test system. However, our results for the strains used in the studies, showed that the *in vivo* test (suckling mice) only detected Coxsackie A virus at an amount of 10^5 TCID₅₀ and Coxsackie B at an amount of 16 TCID₅₀. In contrast, Coxsackie A virus was reliably detected in both Vero and MRC-5 indicator cells at a titer of 10 TCID₅₀. Coxsackie B virus was reliably detected in all 3 indicator cell lines with Vero and HeLa cells being the most sensitive, each detecting 0.1 TCID₅₀. Thus, the indicator cells were 4 logs more sensitive for detecting the strain of Coxsackie A virus used and 2 logs more sensitive for detecting the strain of Coxsackie B virus used.

Interestingly, neither measles virus nor rubella virus were detected in eggs at any titer, even though measles vaccine is normally propagated in chicken embryo fibroblast cultures and the vaccine strain was used in these studies. However, we cannot exclude that attenuating mutations arose during passage to produce the RVS. Historically, rubella virus was propagated in duck embryos, rather than in chicken embryos, but only chicken eggs are used in the routine tests and thus, in this study. Measles virus was detected in all three indicator cell lines, with detection in Vero cells at a titer as low as 0.01 TCID₅₀. Rubella was not readily detected in the 3 indicator cell lines, including the MRC-5 cells in which only the undiluted stock was detected reliably, despite the vaccine strain being grown on WI-38 cells, a similar human embryonic lung cell line. Rubella virus was readily detected on the positive

control RK-13 cell line, though. However, this cell line is not routinely included in the standard test, but it can be used and sometimes is.

Reflecting on the original hypothesis of the study, there were in fact only two viruses that were detected with more sensitivity *in vivo* than *in vitro*. Both VSV and influenza were detected more sensitively in eggs, and VSV was detected more sensitively in post-weaning mice, than in cell culture. Whether inclusion of alternative cell cultures that might detect influenza virus or VSV more sensitively than the standard indicator cells was not studied. However, it is feasible that inclusion of Madin-Darby canine kidney (MDCK) cells might have improved the ability of the *in vitro* test to detect influenza viruses. Consideration needs to be given to whether particular viruses like these two might be potential contaminants of concern for a given cell substrate or vaccine to determine whether it is warranted to continue including the test in eggs in order to not miss one of them. It should be noted that some strains of influenza virus, particularly H3N2, do not propagate well in eggs until they have been adapted for egg growth, and so this enhanced sensitivity may be reflective of the particular strain chosen (an H1N1 strain).

Based on the results from these studies, several conclusions may be drawn. Firstly, the results support consideration of using a broader panel of cell lines for adventitious agent detection, especially if justifying replacement of the *in vivo* tests. Currently, for vaccines made in human or simian cell lines (e.g., MRC-5, Vero), the cell culture tests often use MRC-5, Vero, and HeLa cell lines to encompass the requirement to include a human diploid cell, a monkey kidney cell line, and a cell line of the same species as the cell substrate in the testing. One might question the value of including two human cell lines. However, the data show that MRC-5 and HeLa cells have differing sensitivities (in terms of LOD) for various viruses. For example, MRC-5 cells were more sensitive for detection of echovirus, HSV, and mumps virus, whereas, HeLa cells were more sensitive for detection of adenoviruses, Coxsackie B virus, and rhinovirus.

Secondly, the longer duration (28 days) of testing *in vitro* is supported by the data. In many cases, 14 days of incubation were adequate to detect a positive result at the LOD for the *in vitro* tests. In some cases, only a higher titer was detected by 14 days, and the LOD was improved with the 28-day culture (two 14-day cultures with a blind passage between them). These results suggest that while there should be continued use of the 28day culture period for the *in vitro* tests, decisions might be made on the basis of negative results at 14 days in situations where some level of risk is acceptable, e.g., proceeding with further manufacture. However, lot release for clinical use should continue to rely on the 28-day data.

Thirdly, the longer duration of the suckling mouse assay, which includes a blind sub-passage was not supported by the data. There were no examples where a test or a dilution that gave a positive result did not do so in the original inoculations and required the blind sub-passage into new suckling mice. While this method should permit amplification of an undetectable contaminant that has amplified somewhat in the first passage to achieve detectable levels during the second, this benefit was not seen in these studies. Consideration should be given to refining this test method by reducing the number of animals used by eliminating the blind sub-passage.

Fourthly, the *in vivo* tests may not be as sensitive or as broadly susceptible or otherwise superior to the *in vitro* test, as may have been previously thought. Public reports have been given by testing service providers that in the past 2-3 decades (the GMP “era”), no adventitious viruses have been detected using the *in vivo* tests that were not also detected in the cell culture tests (and the ones that were detected were publicly reported to have been detected in the mouse antibody production test, which is an *in vivo* test, but not the one studied here). Our results suggest that the *in vivo* tests, as routinely performed, are not capable of reliable and sensitive detection of most of the viruses tried, even those expected to be detected by these systems (e.g., Coxsackie viruses; Figure 1).

Based on these public reports and the data reported herein, the value added by the *in vivo* methods can be questioned. While it is true that many viruses do not propagate in cell culture, and thus, the cell culture tests would be incapable of detecting them, we suggest that the adventitious agents of greatest concern are those that will propagate in the production cell substrate, because only those will be of high enough concentrations at harvest or cell banking to: (1) be detectable in the small volumes used in the *in vivo* tests, (2) challenge and perhaps overwhelm the ability of any purification or viral clearance steps in the production process to remain present in the final product, and (3) be a sufficient inoculum in the final product to infect human recipients.

An argument frequently given for continued reliance on the *in vivo* tests is that they can detect unknowns that might not propagate and be detectable in cell culture. This argument may be valid when primary cell cultures or eggs are used for vaccine production; however, if the potentially adventitious virus cannot propagate in cell culture, then the processes of cell expansion and perfusion or batch feeding of production cultures will dilute the potential contaminant. This dilution effect will further reduce a low-level non-amplifying contaminant to such low levels that the probability of a sufficient inoculum being present in the small sample taken from a large bioreactor, wave bag, or other culture vessel and used in the *in vivo* tests will be miniscule. Unlike cell cultures, where the inoculum can amplify from cell to cell and thus, increase the odds of detection, *in vivo*, the inoculum will not readily spread from animal to animal to amplify detection because of the manner in which animals are housed (in groups of five in the case of post-weaning mice although sometimes they are housed in pairs; separately in the case of eggs, and by litter in the case of suckling mice). Only in the case of suckling mice is there a real opportunity for animal-to-animal spread, but in that situation, the pups that die are often cannibalized by the dam and if there is excess death in one but not both litters, often it is concluded that the dam is a poor mother and little proof remains that an spreading adventitious agent would be the cause. Such a result often leads to a retest, where again, the probability of having sufficient concentration in the inoculum into one animal drives the ability to detect the putative contaminant. In suckling mice, these volumes are 0.01 and 0.1 mL i.c. and i.p, respectively. A 0.1 mL sample from even a small 100-liter bioreactor that might be used in investigational vaccine production is only a fraction of one in a million. If large bioreactors (1000L or 10,000L) are used, the detection of a low-level contaminant would become highly unlikely considering the sample volumes used the *in vivo* tests. And only if more than 20% of the animals die or show signs of viral infection from the inoculum will the test be considered to have failed, suggesting the

presence of an adventitious agent. If fewer than 20% of the animals die or show signs of viral infection, the test passes. Thus, only those contaminants that can amplify in the production system are likely to be detectable by the adventitious agent tests, particularly the *in vivo* tests, and these should be detectable by the cell culture test that permits further amplification, if the proper endpoints are used for detection.

Based on our findings, it seems appropriate to suggest considering expanding the panel of cell lines in the *in vitro* test to potentially increase assay sensitivity for some viruses if the *in vivo* methods are reduced or eliminated. Consideration could be given to incubation of indicator cells at lower temperatures (e.g., 33-35°C) to enhance detection of respiratory viruses that propagate better in the lower temperatures of the upper respiratory tract (e.g., rhinoviruses). Furthermore, consideration could be given to optimizing the test for detection of cell-associated viruses. Cell debris may interfere with the test, but may contain the cell-associated viruses that one desires to detect. It might be appropriate to add additional endpoints as well (e.g., IF for specific viruses of greatest concern or microarray or broad PCR-mass spectrometry for broad detection following amplification in culture). Decisions about which cell lines or which endpoints could be added would need to take into account the likely contaminants for the material under test. Thus, a thorough risk assessment should be undertaken to identify potential contaminants in order to rationally guide a proposed testing strategy. For example, if bovine serum or bovine or porcine trypsin were used in the legacy of the cell substrate or viral seed, inclusion of cell lines and endpoints capable of detecting bovine and porcine viruses could be considered in the panel of cell lines used in the *in vitro* testing. In some cases, e.g., for products made in facilities that also make rubella vaccine, RK-13 cells might be considered for inclusion. Although not tested in these studies, when avian cell substrates (CEF or chicken eggs) are used for production (or development of the viral seed), CEF could be incorporated in the panel of cell lines. Consideration must be given to the value added by the *in vivo* tests for a given situation. It may be the case that they are not of sufficient value to justify the use of animals for product safety testing when production is conducted in culture of established cell lines (although use of primary cell cultures might warrant continued use of the test methods). It would be important to acknowledge that changes in regulated tests would require the approval of the relevant regulatory agencies prior to implementation.

To help developers of new assay methods compare the capabilities and performance parameters (such as sensitivity and selectivity) of their methods with the routine methods employed in these studies, the viral stocks prepared for these studies will be made publicly available through the DAIDS Reagent Resource Support Program for AIDS Vaccine Development repository [16]. In addition, protocols may be provided on the methods used to propagate and characterize the viral stocks and to conduct the studies. It was a goal of the project to facilitate method developers and manufacturers wanting to implement the new methods in place of or to supplement the routine tests to have baseline data upon which to judge the relative capabilities and make decisions. It should be noted that these materials are not international standards or reference materials, but are research reagents to be made available to researchers.

Future directions that might be considered to expand the findings reported here would be: to increase the viral families represented, to include variants and other strains of the viruses tested, to include wild-type isolates, to test by spiking the viral stocks into various test sample matrices that might represent actual test samples (e.g., a vaccine virus harvest), to test alternative incubation temperatures that might enhance the ability to detect certain viruses, to optimize for detection of cell-associated viruses, or to increase the number of replicates (to allow a statistical basis for judging a “reliable” LOD) or the parameters studied (e.g., intermediate precision). Also, completing the matrix over every cell of the checkerboard for the *in vivo* tests might be considered. As discussed above, all of these potential factors were outside the scope of the project supporting the studies undertaken.

Another useful future consideration would be to help new method developers outline how they might go about comparing their methods, which are mostly molecular genomics methods, to the routine tests that have infectivity and pathogenicity endpoints. While manufacturers and regulators are most concerned about “demonstrable viable” microbial agents that might represent a safety risk, impurities are also a concern. Molecular genomic or transcriptomic methods, like deep sequencing, mass spectrometry following broad-spectrum PCR, and microarrays, are capable of detecting both intact and fragmented viral nucleic acids (NA). These NA may or may not represent a risk and may only be impurities of a fragmented residuum of an inactivated contaminant that may no longer be hazardous. Depending on how they are applied, these new methods may not readily distinguish between inactive sequence copies and those resulting from amplification of infectious viruses. How to compare genome copies with TCID₅₀ or PFU is unclear in most situations. This same concern has been raised in considering using PCR to detect mycoplasma contamination and much consideration has been given to how best to do this. Whether similar approaches could be taken for viruses, which have a much greater breadth of characteristics than do mycoplasmas [e.g., ability to be detected in the routine tests, nucleic acid (NA) content/organism, type of NA (DNA or RNA as well as single or double-stranded NA)] may escalate this challenge further. Consideration should be given to using standard viral stocks that have been characterized for both infectivity titer and genomic copy numbers as a basis for comparison or using the same stocks for each assay method and simply comparing the highest dilution at which each demonstrates detection.

In summary, the results of the studies reported here provide the first systematic data on comparing the breadth of detection and sensitivities of the *in vivo* adventitious virus tests and the *in vitro* tests. The data point to limitations with the *in vivo* tests and suggest ways that the *in vitro* tests might be refined or improved, such as the potential inclusion of additional cell lines. Combining expanded *in vitro* testing with the new generation of molecular methods for hybrid assays might provide, in time, a pathway for implementing the 3 R's, i.e., the reduction, refinement, or replacement of the *in vivo* tests. Although the methods used for decades have been largely successful at keeping contaminated vaccines off the market, in the few cases that they have failed, the contaminants have not posed a risk to human health [17]. Nonetheless, in the spirit of Quality by Design, it is incumbent upon us to seek to continuously improve our methods to assure safe and pure vaccines are available to protect and promote the public health.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

ACKNOWLEDGEMENTS

The authors would like to acknowledge Kathryn Butler for initiation of this project, Sveta Sherbaty, Amy Bennett, and Marci Henkin for excellent technical assistance in the preparation and characterization of the research virus seeds, Cheryl Harper and Christine Nogier for critical data review, Michael Nicholson for thorough Quality review, and Michelle Walker for project coordination. We also thank Dr. Barry Rosenblatt for many helpful discussions of the study design and for presentation of preliminary data at public meetings, and Christine Simko for excellent assistance during preparation of this manuscript. Further, we would like to acknowledge Jack Hill for his budget management and assistance in this project. Dr. Keith Peden served on the expert panel that guided the project initially and has reviewed the manuscript and we thank him for his advice and guidance.

This project has been funded in whole or in part with Federal funds from the National Institute of Allergy and Infectious Diseases, National Institutes of Health, Department of Health and Human Services, under Contract No. HHSN266200400045C.

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Fig. 1. Comparison of Limits of Detection for *In Vivo* and *In Vitro* Assays for Model Adventitious Viral Agents

LOD values observed during *in vivo* (left panel) and *in vitro* (right panel) testing of each of the viruses used in this study. Shorter bars indicate lower sensitivity (i.e., higher LOD). In those cases where additional cell lines were used for *in vitro* tests and they provided greater sensitivity (Ad41, BVDV, and rubella virus), the LODs observed using the standard three cell lines and also using the additional cell line are represented as a stacked bar. UN = undiluted, ND = not detected, NT = not tested.

Table 1

Virus Strains and Cell Lines Used to Prepare Research Virus Stocks

Virus Family	Virus (Strain)	Abbreviation	Virus Source (CRL or Vendor Number)	Cell Line	ATTC Cell Line
<i>Adenoviridae</i>	Adenovirus 5	Ad5	1-647-96	A549	CCL-185
	Adenovirus 41	Ad41	1-240-176	HEK293	CRL-1573
<i>Herpesviridae</i>	Simian Cytomegalovirus	sCMV	2-647-169	BS-C-1	CCL-26
	Herpes Simplex Virus Type 1 (MacIntyre)	HSV	1-575-94	Vero	CCL-81
<i>Polyomaviridae</i>	Simian Virus40	SV40	3-1240-86	Vero	CCL-81
<i>Flaviviridae</i>	Bovine Viral Diarrhea Virus (NY-1)	BVDV	647-59-111502	BT	CRL-1390
<i>Orthomyxoviridae</i>	Influenza A (A/PR/8/34)	Flu	ATCC VR-1469	MDCK	CCL-34
<i>Paramyxoviridae</i>	Measles (Edmonston)	Not applicable	1-647-74	Vero	CCL-81
	Mumps (Enders)	Not applicable	1-1240-150	Vero	CCL-81
	Bovine Parainfluenzavirus Type 3	BPIV-3	3-1017-2	Vero	CCL-81
	Coxsackie Virus A16	CAV	Zeptomatrix 0810107CF	Vero	CCL-81
<i>Picomaviridae</i>	Coxsackie Virus B3	CBV	1-1240-177	LLC-MK2	CCL-7
	Echovirus 11 (Gregory)	Echo	1-240-181	LLC-MK2	CCL-7
	Rhinovirus 2	Rhino	2-1178-82	HeLa	CCL-2
<i>Rhabdoviridae</i>	Vestibular Stomatitis Virus (Indiana)	VSV	4-705-111	Vero	CCL-81
<i>Togaviridae</i>	Rubella virus (M-33)	Not applicable	1-1178-189	BS-C-1	CCL-26

Table 2

Study Design for *In Vitro* and *In Vivo* Adventitious Virus Testing

Virus	Stock Titer	In Vitro Assays					In Vivo Assays		
		MRC-5	HeLa	Vero	Other	Endpoint Observations	Post-weaning Mice	Suckling Mice	Eggs
Adenovirus 5	3.0×10 ⁸ TCID ₅₀ /mL	X	X	X	A549	CPE ^a , HAD ^b			
Adenovirus 41	2.0×10 ⁵ TCID ₅₀ /mL	X	X	X	A549	CPE, HAD			
Bovine Parainfluenzavirus 3	6.3×10 ⁷ TCID ₅₀ /mL	X	X	X		CPE, HAD			X
Bovine Viral Diarrhea Virus	3.0×10 ⁷ FAID ₅₀ ^c /mL	X	X	X	BT	CPE, IF ^d	X	X	
Coxsackie A16 virus	6.3×10 ⁶ TCID ₅₀ /mL	X	X	X	RD	CPE, HAD		X	
Coxsackie B3 virus	3.0×10 ⁷ TCID ₅₀ /mL	X	X	X		CPE, HAD		X	
Echovirus 11	2.0×10 ⁷ TCID ₅₀ /mL	X	X	X		CPE, HAD		X	
Herpes Simplex Type 1	9.5×10 ⁶ PFU/mL	X	X	X		CPE, HAD	X	X	
Influenza A virus	6.3×10 ⁷ TCID ₅₀ /mL	X	X	X		CPE, HA			X
Measles virus	9.3×10 ⁵ TCID ₅₀ /mL	X	X	X		CPE, HAD			X
Mumps virus	1.3×10 ⁶ TCID ₅₀ /mL	X	X	X		CPE, HAD			X
Rhinovirus 2	1.3×10 ³ TCID ₅₀ /mL	X	X	X		CPE			
Rubella virus	1.3×10 ⁴ TCID ₅₀ /mL	X	X	X	RK-13	CPE, HA ^e			X
Simian Cytomegalovirus	3.0×10 ⁸ TCID ₅₀ /mL	X	X	X	BS-C-1	CPE, HAD			
SV40	7.1×10 ⁷ PFU/mL	X	X	X		CPE, HAD			
Vesicular Stomatitis Virus	1.0×10 ⁹ PFU/mL	X	X	X		CPE	X	X	X

Shaded boxes designate combinations that were not done

^a Cytopathic effect^b Hemadsorption^c Fluorescent antibody infectious dose 50^d Immunofluorescence^e Hemagglutination

Table 3Test systems used for *In Vivo* Adventitious Virus Assays

Host	Route/Dose	Test Material	# per group	Post Inoculation Observation Period
Post Weaning Mice (3-4 wks, 15-20g)	IP/ 0.5mL IC/ 0.03mL	Virus	20	21 days
Primary Inoculation Suckling Mice (< 24 hours old)	IP/ 0.1mL IC/~0.01mL	Virus	20	14 days
Passage Suckling Mice	IP/ 0.1mL IC/~0.01mL	Homogenate from primary inoculation group	20	14 days
Embryonated Chicken Eggs (6-7days old for yolk sac inoculation, 10-11 days old for allantoic route)	Primary Inoculation Allantoic Fluid/ 0.5mL	Virus	10	3 days
	Passage Allantoic Fluid/ 0.5mL	Pooled Allantoic fluid from Primary Inoculation Group	10	3 days
	Primary Inoculation Yolk Sac / 0.5mL	Virus	10	9-12 days
	Passage Yolk Sac / 0.5mL	Yolk Sac homogenate from primary inoculation group	10	9-12 days

Table 4*In Vitro* Limit of Detection of Research Virus Stocks

Virus	Vero		MRC-5		HeLa		Other ^d	
	CPE	HA/HAD/IF ^b	CPE	HA/HAD/IF ^b	CPE	HA/HAD/IF ^b	CPE	HA/HAD/IF ^b
Adenovirus 5								
Adenovirus 41								
BVDV								
BPIV-3								
Coxsackie A16								
Coxsackie B3								
Echovirus 11								
Influenza A								
HSV								
Measles								
Mumps								
Rhinovirus 2								
Rubella								
Simian CMV								
SV40								
VSV								

^a Refer to Table 2 for the "other" cell lines used in each specific case
^b All virus-infected cultures were tested for hemadsorption activity except BVDV (immunofluorescence), influenza A and rubella (hemagglutination) and rhinovirus 2 and VSV (CPE only)
^c Detection by immunofluorescence
^d Not tested

Color Key: Infectious doses inoculated in 0.5 mL per well

0.001 ID
0.01 ID
0.1 ID
1.0 ID
10.0 ID
100.0 ID
1,000.0 ID
100,000.0 ID
Undiluted
Undetected

Table 5

Detection of Viruses in Animal Hosts: Inoculation of Undiluted Virus Stocks

Inoculum			% Mortality ^a					
			Mice		Eggs			
Virus	Titer/mL	Units	Suckling ^b	Post-Weaning	Allantoic Fluid		Yolk Sac	
					Primary	Passage	Primary	Passage
BPIV- 3	6.3×10 ⁷	TCID	NT ^c	NT	0	0	0	10
BVDV	3.0 ×10 ⁷	FAID	0	5	NT	NT	NT	NT
Coxsackie A16 virus	6.3 ×10 ⁶	TCID	30	NT	NT	NT	NT	NT
Coxsackie B3 virus	3.0 × 10 ⁷	TCID	100	NT	NT	NT	NT	NT
Echovirus 11	2.0 × 10 ⁷	TCID	5	NT	NT	NT	NT	NT
HSV	9.5 × 10 ⁶	PFU	100	100	NT	NT	NT	NT
Influenza virus	6.3 × 10 ⁷	TCID	NT	NT	100	NT	100	NT
Measles virus	9.3 × 10 ⁵	TCID	NT	NT	0	0	0	10
Mumps virus	1.3 × 10 ⁶	TCID	NT	NT	0	NT	80	NT
Rubella virus	1.3 × 10 ⁴	TCID	NT	NT	0	10	0	0
VSV	1.0 × 10 ⁹	PFU	100	100	90	NT	100	NT

^aPercent mortality observed among 10 eggs or 20 post-weaning mice or at least 20 suckling mice^bAlthough passage in suckling mice was performed for dilutions that did not score positive by day 14, no additional mortality was noted and this information is not indicated in the table as a consequence^cNT- Not tested

Table 6

Inapparent Virus Studies: Day Post Inoculation When Animal Host Mortality Exceeded 20% When Inoculated with Undiluted Virus Stocks

System	Virus	DPI	% Mortality
Suckling Mice	VSV	2	100%
	HSV	3	100%
	CAV	7	30%
	CBV	2	100%
Post-Weaning Mice	VSV	2	85%
	HSV	3	75%
Eggs - Yolk Sac Route	VSV	5	100%
	Mumps	8	60%
	Influenza	3	100%
Eggs - Allantoic Route ^a	VSV	3	100%
	Influenza	3	100%

^aEmbryonated eggs not examined on day 2