



Historical evaluation of the *in vivo* adventitious virus test and its potential for replacement with next generation sequencing (NGS)

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ABSTRACT

The Consortium on Adventitious Agent Contamination in Biomanufacturing (CAACB) collected historical data from 20 biopharmaceutical industry members on their experience with the *in vivo* adventitious virus test, the *in*

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Viral safety
In vivo virus test
In vitro virus test
 Virus contamination
 Broad spectrum virus detection

in vitro virus test, and the use of next generation sequencing (NGS) for viral safety. Over the past 20 years, only three positive *in vivo* adventitious virus test results were reported, and all were also detected in another concurrent assay. In more than three cases, data collected as a part of this study also found that the *in vivo* adventitious virus test had given a negative result for a sample that was later found to contain virus. Additionally, the *in vivo* adventitious virus test had experienced at least 21 false positives and had to be repeated an additional 21 times all while using more than 84,000 animals. These data support the consideration and need for alternative broad spectrum viral detection tests that are faster, more sensitive, more accurate, more specific, and more humane. NGS is one technology that may meet this need. Eighty one percent of survey respondents are either already actively using or exploring the use of NGS for viral safety. The risks and challenges of replacing *in vivo* adventitious virus testing with NGS are discussed. It is proposed to update the overall virus safety program for new biopharmaceutical products by replacing *in vivo* adventitious virus testing approaches with modern methodologies, such as NGS, that maintain or even improve the final safety of the product.

1. Introduction

The contamination of biologic products, such as recombinant proteins (e.g., antibodies) and viral vaccines, and their manufacturing processes with adventitious viruses is rare. A study published in 2020 by the Consortium on Adventitious Agent Contamination in Biomanufacturing (CAACB) reported 26 such events, relative to thousands of recombinant manufacturing batches per year, between 1985 and 2020 [1]. To our knowledge, none of these events, which do not include egg-based vaccines and plasma products, have led to the transmission of an adventitious virus to a patient, with all but one contamination detected before any drug substance or drug product was released [1,2]. This safety record is largely attributed to the complementary approach to viral safety outlined in ICH Q5A and implemented by biologic manufacturers relying on the selection of starting materials and raw materials that are of low risk for potential adventitious viral contamination, testing for viral contaminants that may be present in starting materials or that were unintentionally introduced in the manufacturing process, and ensuring robust viral clearance to remove or inactivate both known process impurities, such as endogenous retrovirus-like particles, and undetected viral contaminants [3].

As noted, a key pillar of viral safety in the development and manufacture of biologic products from human or animal cells is testing for the presence of adventitious viral agents [3]. This ranges from testing and characterization of the master and working cell banks to ensure that the production cells themselves are free of detectable potential viral contaminants to in-process and lot-release tests to detect adventitious viruses unintentionally introduced into the process. Broad spectrum and agnostic detection assays are used to ensure that viral contaminants have the potential to be detected [3,4]. Two of the main viral detection assays used for this purpose are the *in vitro* adventitious virus test and the *in vivo* adventitious virus test. The *in vitro* virus test involves inoculating the test article onto indicator cells chosen, based on a risk assessment, for their potential to detect viral contaminants that may be present in the raw materials, propagate in the host cell line, or are a potential risk to patients. The cells are observed for indicators of infection, such as cytopathic effect (CPE), hemagglutination (HA), hemadsorption (HAD), or viral antigen immunofluorescence [3–6]. Similarly, the *in vivo* adventitious virus test involves inoculating the test article into living animals and observing them for illness or mortality or testing the animal tissue itself directly for the presence of virus. Common animals that are/were used in the *in vivo* adventitious virus test include both adult and suckling mice, guinea pigs, and embryonated chicken eggs as well as hamsters, mice, and rats for the production of virus specific antibodies [4,5]. Both the *in vivo* adventitious virus test and the *in vitro* virus test are currently recommended by guidance documents as key tools to ensure the continued viral safety of biologic products [3,5] and are also listed in the US Code of Federal Regulations as required for certain vaccines [7–10]. Specifically, the *in vivo* adventitious virus test is used to test cell banks for both vaccines and recombinant products as well as viral seeds for vaccines; the *in vitro* virus test is used to test cell banks for both vaccines and recombinant products, viral seeds for

vaccines, and pre-harvest bulk for both vaccines and recombinant products.

Despite their broad use and history in contamination control of biotech products, there is a growing interest to explore new virus detection technologies and move away from the *in vivo* adventitious virus test [11]. The European Pharmacopoeia has articulated guidance to this effect [12,13] and several industry initiatives are currently exploring the potential of high-throughput sequencing (HTS), also known as Next Generation Sequencing (NGS) or Massively Parallel Sequencing (MPS), for broad spectrum virus detection [14–18]. This is partially driven by a desire to reduce the number of animals used, as seen in the replace, reduce, refine strategy articulated by the EMA [11], previous failures of traditional adventitious virus tests to detect viral contaminants [2,19], the need to develop rapid adventitious agent testing approaches for emerging products [1], and concern over the *in vivo* adventitious virus test's ability to detect viruses as compared to other adventitious virus testing approaches. To the latter point, a publication from Gombold et al. in 2014 compared the performance of the *in vivo* adventitious virus test to the *in vitro* virus test for the detection of 11 different viruses [4]. The *in vitro* virus assay detected all 11 viruses tested and was overall more sensitive than the *in vivo* adventitious virus test at detecting nine of the viruses tested. In comparison, the *in vivo* adventitious virus test, while more sensitive at detecting VSV and influenza in their suitable animal model (eggs for influenza, and mice/eggs for VSV), was unable to detect five of the viruses. Aside from this study, there is little publicly available information to guide biopharmaceutical manufacturers in assessing when, and in what situations, it may be appropriate to replace the expected existing broad spectrum *in vivo* adventitious virus test with an alternative method.

To partially address this gap, the CAACB collected historical data from 20 biopharmaceutical industry members in Spring of 2021, through a 36-question survey, on their experience with the *in vivo* adventitious virus test, the *in vitro* virus test, and the use of next generation sequencing for viral safety. The goal of this work was to provide a retrospective historical review of the traditional virus detection assays to guide biopharmaceutical manufacturers in conducting viral risk assessments and assessing the value of such assays. The CAACB also collected information on its members' current status in the use or exploration of NGS as a viral safety tool. Survey participants include both biopharmaceutical manufacturers and contract testing laboratories that perform virus testing. In this paper, the findings from the survey data are presented. Considerations for replacing a biological assay with a molecular method, current risks to replacing the *in vivo* adventitious virus test with NGS, and some potential challenges to implementing NGS in the industry are also discussed.

2. The *in vivo* and *in vitro* virus assays

Both the *in vivo* adventitious virus test and the *in vitro* virus test have been in use for more than 50 years [20]. They were originally developed as tools to detect potential viral contaminants in vaccines and are now widely used in the biotechnology industry [1,4]. This wide use was

confirmed by the CAACB survey. The CAACB asked its members for what purpose they are currently using *in vivo* adventitious virus testing in their manufacturing process and product development activities. The results are shown in Fig. 1. From these data, the *in vivo* adventitious virus test is currently widely used, with 94% of respondents using some form of the assay for cell line characterization and 50% of respondents using the assay for virus seed testing and lot release. In the case of viral seed testing, this was performed for vaccines, viral vectors, helper virus (for viral vector production), and oncolytic viruses.

As seen in Fig. 2, the most commonly used indicator animals for *in vivo* adventitious virus test are adult mice, suckling mice, and embryonated chicken eggs (each with 94% of respondents), followed by viral antibody production tests in hamsters, mice, or rats (HAP/MAP/RAP), and guinea pigs. No respondents reported using rabbits.

Since 2000, CAACB members have completed more than 10,000 *in vivo* adventitious virus tests, where a test was defined as a single sample inoculated into a single species at one concentration. These tests used more than 84,000 animals (including embryonated chicken eggs). Additionally, over that same time, CAACB members have performed more than 67,000 *in vitro* virus tests using indicator cell lines. It is important to note that these numbers are conservative estimates and are likely undercounted. Several CAACB members were unable to accurately determine the number of adventitious virus tests performed by their company or the number of animals used in those tests. Additionally, CAACB members comprise only a small fraction of the biotechnology industry (~14%), with more than 140 biopharma companies participating in the 2021 Annual Biomanufacturing Capacity and Production Report [21]. Therefore, the actual number of assays performed, and animals used, is likely much higher than what is reported here.

2.1. Reliability of *in vivo* and *in vitro*

The primary value of the *in vivo* adventitious virus test currently claimed in the literature and guidance is its ability to detect potential viral contaminants that are not detectable in other tests, such as viruses that cannot be propagated in cell culture. For example, and as previously introduced, the 2014 publication by Gombold et al. provided experimental evidence that the *in vivo* adventitious virus test was more sensitive than the *in vitro* virus assay to detect VSV and influenza as those viruses actively and symptomatically replicate in the tested animal model [4]. However, to our knowledge, there is no public report of historical *in vivo* adventitious virus test results evaluating whether this is true in practice. To that end, CAACB members were asked if, over the lifetime of their using the *in vivo* adventitious virus test, they had experienced a true positive *in vivo* adventitious test that was NOT also detected in another supporting assay. All responding CAACB member companies indicated that, in their experience, **this had not happened**. Of the three virus positives obtained by the *in vivo* adventitious virus tests reported to the CAACB, all were also detected by the *in vitro* virus

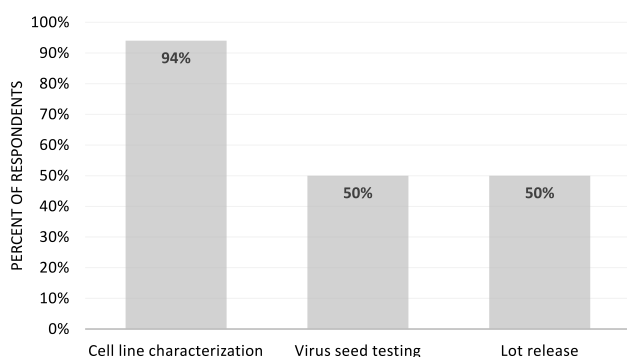


Fig. 1. Percent of respondents that utilize *in vivo* adventitious virus testing for the indicated purpose (number of responding companies equals 20).

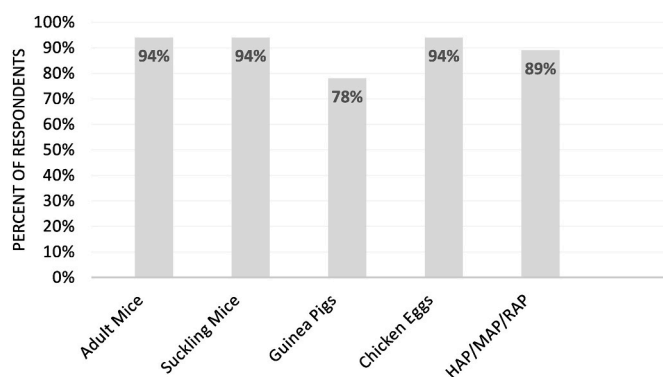


Fig. 2. Percent of respondents that perform *in vivo* adventitious virus assays with the indicated animals. The number of responding companies equals 20.

test. While it was often difficult for members to collect all historical data related to the *in vivo* adventitious virus assay, the information reported to the CAACB, together with published reports indicative of the *in vivo* adventitious virus test missing a viral contaminant [2,19] raises questions as to the continued use of the *in vivo* adventitious virus test as a tool for viral safety in biologics manufacturing. This is particularly true when compared to other broad spectrum virus testing [2,4,14].

In addition to a test's ability to detect potential contaminants, test reliability is a key component of evaluating the suitability of a test both from a safety and a business perspective. All tests have the potential to experience false positives (a positive result from a sample that does not contain a virus), produce a negative result from a sample that does contain a virus, or need to be repeated due to an error in the test or its controls. To further understand the value provided by the *in vivo* adventitious virus test, the CAACB also collected data from its members reviewing this historical reliability.

In the case of the *in vivo* adventitious virus test, 21 tests were reported to the CAACB to have initially given a positive test result that was later determined to be a false positive. The detailed distribution of false positives reported per animal type is shown in Table 1. The *in vivo* adventitious virus false positive tests were reported to occur in five out of seven animal species utilized, except guinea pigs and rats. The data do not provide sufficient evidence to claim that one animal species or the *in vivo* adventitious virus test is more susceptible to false positives than another.

Each false positive must initially be treated as a true positive event with a subsequent investigation conducted to confirm the contamination or to determine that it is, in fact, a false positive. As seen in Fig. 3, 10 false positive events in the *in vivo* adventitious virus assay required one to three months to complete the follow-up investigation, including conducting repeat testing as needed. However, there was one reported event that took longer than 12 months to resolve.

In addition to false positives, negative events, where the *in vivo* adventitious virus test gave a negative result for a sample that was later found to be positive for virus, were also reported to the CAACB. There were two confirmed negatives (one in mice and one in embryonated eggs) that were later determined to be positive via the *in vitro* virus test and one organization that indicated they had experienced multiple negatives that were later determined to be positive but were unable to identify the exact number.

It may also be necessary to repeat the *in vivo* adventitious virus test to obtain valid results, most commonly due to technical issues in the original test, such as injection site trauma, injury due to improper handling, cannibalism, or non-viable eggs. CAACB members reported that more than 21 *in vivo* adventitious virus tests needed to be repeated. Not all instances of repeat *in vivo* adventitious virus tests were able to be documented. Therefore, the actual number of repeat assays experienced by CAACB members is likely larger than that reported here. The time it takes to repeat an *in vivo* adventitious virus test depends on the animal

Table 1
Number of false positives reported to the CAACB per animal species.

	Adult Mice	Suckling Mice	Eggs	Guinea Pigs	HAP	MAP	RAP
False Positives	2	4	5	0	2	7	0

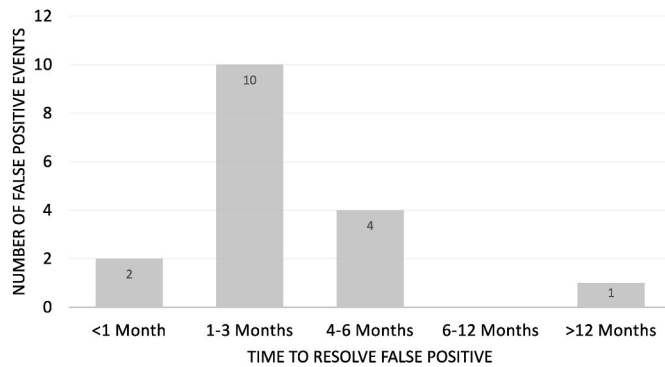


Fig. 3. Distribution of length of time required to resolve *in vivo* adventitious virus test false positive events.

model and the step at which the repeat testing is performed. It can take as little as 18 days to several months. CAACB members reported that 37% of repeat assays were completed in less than one month and the remaining 63% took an additional one to three months to complete.

In addition to understanding the reliability of the *in vivo* adventitious virus test, it was important to further understand the reliability of the *in vitro* virus test. Previous CAACB work has looked at the use of the *in vitro* virus test as a lot release test for biologics manufacturing [1]. In that work, the *in vitro* adventitious virus test successfully detected a viral contaminant in 11 events which occurred under current Good Manufacturing Practice (cGMP) conditions. However, the same study also reported four cases where the *in vitro* adventitious virus test, as a lot release test, was negative for a sample that contained virus [1]. The scope of that previous work was limited to manufacturing. Since then, CAACB members were asked about their experience with negatives in the *in vitro* virus test that later were found to contain virus, including cell line characterization. While the exact number of cases was unable to be reported, a minimum of two additional cases were reported to the CAACB where the *in vitro* virus test was negative but the sample was later determined to be positive from an alternative assay. In total, the *in vitro* virus test has experienced more than six such cases during manufacturing and cell line characterization.

Table 2 compares the false positive, negative for virus containing samples, and repeat rates for the *in vivo* adventitious virus test and the *in vitro* virus test. While conducting nearly seven times more *in vitro* virus tests, based on the data reported to the CAACB, the *in vivo* adventitious virus test has false positive and false negative rates that are 11.7 times and three times, respectively, higher than those of the *in vitro* virus test.

2.2. Alternatives are needed

The data presented above highlight the limitations of current *in vivo*

Table 2
Summary of *in vivo* adventitious virus and *in vitro* virus test reliability.

	<i>In vivo</i> adventitious virus test	<i>In vitro</i> virus test
Number of tests	>10,000	>67,000
False positives (#/%)	>21/0.21%	>12/0.018%
Negative for virus containing samples (#/%)	>3/0.03%	>6/0.009%
Repeats (#/%)	>21/0.2%	NA

and *in vitro* viral detection tests widely used in biologics manufacture. Further, as noted above, despite its wide use in the industry, based on the data collected by the CAACB, **the *in vivo* adventitious virus test has not ever detected a viral contaminant that was not also detected in another companion assay.** While this highlights the importance of a multifaceted approach to viral safety, it also questions the value of the continued use of the *in vivo* adventitious virus test, which has used more than 84,000 animals from CAACB member companies alone since 2000. Further, compared to the *in vitro* virus test, the *in vivo* adventitious virus test has a higher rate of both false positives and false negatives, the former which can take months to resolve. Additionally, there are biotherapeutic products for which one or both of the *in vivo* adventitious virus test or the *in vitro* virus test cannot be performed, such as for live viral products with no neutralizing antibody or for products that require rapid release, to name a few. These data support the consideration and need for alternative broad spectrum viral detection tests that are faster, more sensitive, more accurate, more specific, and more humane. Therefore, it is proposed to update the overall virus safety program first for new biopharmaceutical products by replacing the *in vivo* adventitious virus testing approaches with modern methodologies, such as NGS, that may even improve the final safety of the product. Further, in some cases it may be justified to reduce or remove *in vivo* adventitious virus testing altogether *without* implementing a replacement test. This decision should be driven by a risk assessment and should consider the prior history of the cell substrate (e.g., prior characterization and the use of platform manufacture derived from the same parental cell line). This approach is in line with information previously published by the European Pharmacopoeia [12,13].

3. Next generation sequencing as a potential alternative

One potential alternative to the *in vivo* adventitious virus test is based on next generation sequencing (NGS). Next generation sequencing typically involves sequencing millions of short or long nucleic acid sequences in parallel and mapping those sequences, or larger assembled contigs, against genomic databases to identify the source of the nucleic acid. In 2010, NGS detected a previously undetected viral contaminant in an attenuated live viral vaccine [2]. In this case, the viral contaminant was found to not present a safety risk for patients—it was present since the initial stages of product development and in manufactured lots used in clinical trials [22]. Per dose, the viral DNA was reported to be present at 6–7 log(10) [23] of infectious virus [24] implying that the detection of this contaminant was missed by traditional testing such as the *in vivo* adventitious virus test and the *in vitro* virus test. Shortly thereafter, a previously unidentified virus in a common insect cell line used in bio-production was also discovered by NGS [19]. Since then, there has been growing interest in the use of NGS as a tool for viral detection in the biopharmaceutical industry. This is seen in the formation of the PDA Advanced Virus Detection Technology Interest Group (AVDTIG) [14] and subsequent publications from that effort [15–18], as well as in the recent conferences on next generation sequencing for adventitious virus detection in biologics for humans and animals [25,26]. Specifically, NGS has a number of advantages as a viral detection tool: it can be used as an agnostic method with regards to the sequences detected and is broad spectrum, seems to show comparable or better sensitivity to the *in vivo* virus test [27], and simultaneously detects and identifies the contaminant.

Given the potential of NGS as a broad-spectrum virus detection tool, there is interest in using NGS in the biotechnology industry. As seen in Fig. 4, of CAACB member companies that manufacture biologics, 81% of

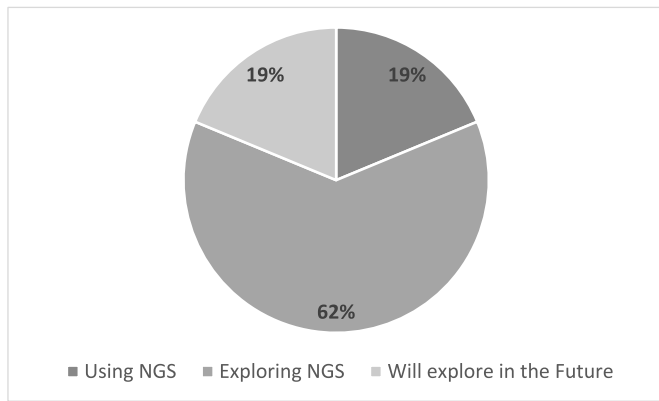


Fig. 4. CAACB companies that are currently using NGS as a viral safety tool, exploring NGS as a viral safety tool, or planning to explore NGS in the future.

respondents were already using or exploring NGS as a viral safety tool. The remaining 19% of respondents indicated plans to explore NGS as a viral safety tool in the future.

Further, there is interest in using NGS to replace or supplement existing adventitious virus assays, where supplementation refers to performing NGS in addition to existing testing. Fig. 5a shows that, of the CAACB companies surveyed, 25% are actively evaluating replacement, 63% are actively evaluating both replacement and supplementation, and 6% are actively evaluating supplementing existing adventitious virus assays with NGS. Fig. 5b also shows that 69% of respondents are exploring replacement and that 50% of respondents are exploring supplementing the *in vivo* adventitious virus assay with NGS. However, there is also interest in replacing (31% of respondents) and supplementing (63% of respondents) the *in vitro* virus test with NGS. These data show a clear desire by the biotechnology industry to replace both the *in vivo* adventitious virus test (69%) and the *in vitro* virus test (31%) with NGS.

Fig. 6 displays the percentage of responding CAACB companies that indicated a specific motivation for exploring the replacement or supplementation of the adventitious virus tests shown in Fig. 5.

While there is clear alignment in the goal to reduce animal use by moving to NGS, the motivations in Fig. 6 point to a broader range of reasons than the reduction of animals alone. First and foremost, 75% of respondents indicated that NGS is expected to have greater breadth of detection and 38% of respondents indicated that NGS is expected to have greater sensitivity than the *in vivo* adventitious virus test, thereby ensuring a safer final product. This point is supported by reports demonstrating the ability of NGS to detect viral contaminants that were missed by other assays [2,19] and by recent reports comparing NGS

sensitivity to that of the *in vivo* virus test [27].

Seventy five percent of respondents also indicated that speed was a major motivation for pursuing NGS as a replacement for existing virus detection assays. The length of time to perform the *in vivo* adventitious virus test will depend on the animal model chosen, ranging from a minimum of five days for embryonated chicken eggs (depending on the guidance followed) and 28 days for suckling mice to 42 days for guinea pigs [5]. Additionally, the times listed are those to perform the *in vivo* incubation, which cannot be improved, and do not include additional time for review and assessment of the results. In comparison, the time to perform sequencing, bioinformatic analysis, and follow-up expert review for NGS depends on the specific application, approach, and if the sequencing is performed in-house or is outsourced. To that end, CAACB members report this can take as little as seven days to as long as 45 days, though the exact timing depends on the specific use of the technology and its aims. While there is significant variability in the time to perform NGS analysis, some reported timelines are already faster than the *in vivo* adventitious virus test. Additionally, technological development is advancing rapidly, both in sequencing and in bioinformatic analysis, and opportunities to shorten the time for analysis will present themselves and are being explored. As an example, there is at least one patent demonstrating agnostic detection of adventitious viruses in cell culture operations using the Oxford Nanopore MinION in less than one day [28].

Should the *in vivo* adventitious virus test return a positive result, the presence of the viral contaminant must be confirmed and the identity determined. In this situation, the *in vivo* adventitious virus test (or the *in vitro* virus test) may be repeated to confirm the initial result, doubling the testing time, or alternative assays may be employed. Of the alternative assays, NGS may also be used as it will both confirm and identify the potential contaminant. Follow-up investigations for positive test results are time consuming, as seen in Fig. 3 where the time to identify a false positive in *in vivo* testing was reported. The mode of the duration to resolve a false positive was 1–3 months, with the maximum resolution time taking more than a year.

In the case of an NGS positive result, the follow up investigation may be more straight forward as the contaminant, or relevant contaminating nucleic acid sequences, will have also been identified at the same time it was detected. Depending on the NGS methodology used, the sequencing may provide evidence supporting viral replication (such as detection of viral RNA transcripts [29]). Otherwise, a follow up confirmation study will likely be required to determine the presence of a replication competent contaminant. As the identity is already known, follow up studies can be performed in a more targeted manner and a much shorter time frame (e.g., using PCR).

Finally, it is noted that when the survey was completed, four CAACB members reported formally interacting with regulatory authorities around the use of NGS, with three including NGS in investigational new

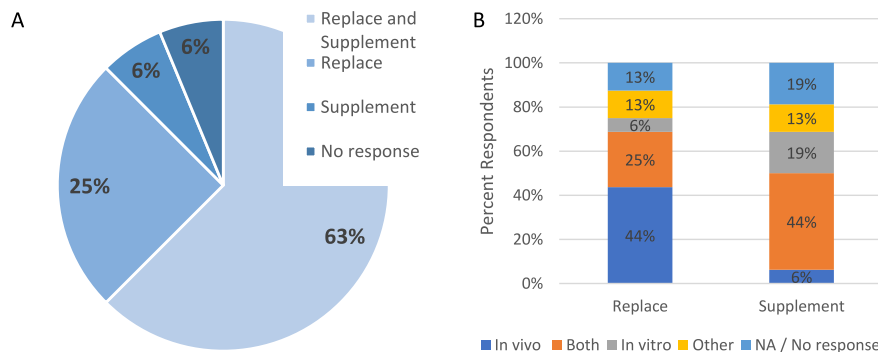


Fig. 5. A) Percent of CAACB companies that are looking to replace and supplement, replace, or supplement existing adventitious virus testing with NGS. B) Breakdown of adventitious virus assays that CAACB companies are aiming to replace or supplement with NGS. Note, some respondents intend to both replace and supplement certain assays depending on the specific application. Number of respondents = 20.

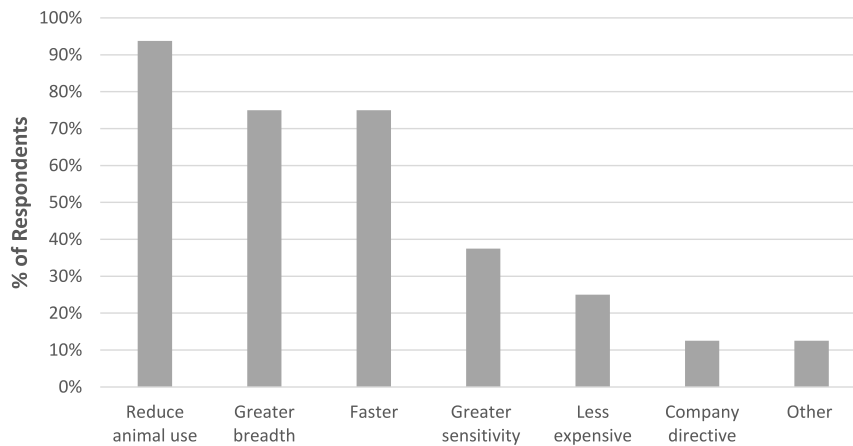


Fig. 6. Percent of CAACB manufacturers who stated a specific motivation for replacing or supplementing existing adventitious virus tests with NGS. NOTE: respondents could select more than one reason for pursuing the replacement or supplementation of existing adventitious virus assays with NGS.

drug regulatory filings. An additional six CAACB members have not yet interacted with the regulators formally, but expect to do so in the near future.

4. Considerations on the replacement of a biological assay with NGS

A key question that must be answered when looking to replace an existing adventitious virus test with NGS is “what information is needed to justify the use of an alternative viral safety test?” As a starting point, existing guidance, previous industry experience in replacing biological assays with molecular assays, and literature data should be considered.

In terms of current guidance, recent communications from regulatory agencies regarding the use of NGS recognizes the advantages to be gained from using alternatives to the *in vivo* adventitious virus test. In Europe, the European Pharmacopoeia has provided guidelines for vaccines outlining approaches for using broad molecular methods as alternatives to *in vivo* testing (such as suckling mice and embryonated eggs) [12] as well as indicating that broad molecular methods, such as NGS, are accepted as alternatives to both the *in vivo* adventitious virus test and the *in vitro* virus test, depending on a risk assessment [13,30]. A common requirement for using molecular methods as a replacement is that the sensitivity and specificity must at least match that of the existing assay, as validated by testing against a panel of representative viruses. Because the readout from molecular methods (the presence of viral genome) is different from the readout in animal methods (typically a visible impact on animal health or mortality), this comparison is complex and may not be possible. Therefore, it may be necessary to justify the use of an alternative assay, such as NGS, in place of an existing assay without a direct head-to-head comparison. Instead, it will be necessary to demonstrate the suitability of the new assay for the proposed purpose and support this justification by a risk assessment. Such an approach is currently envisaged in Ph. Eur. 5.2.14 [12]. More broadly, as of Q4, 2022 the revised ICH Q5A guideline on *Quality of biotechnological products: viral safety evaluation of biotechnology products derived from cell lines of human or animal origin* was released for public comment and described the applicability of NGS as a replacement for animal methods. Further, it should be noted that a head-to-head comparison between a new method and an animal-based test is not aligned with the goals of replace, reduce, and refine (or the 3Rs).

Outside the realm of viral contamination, mycoplasma detection is currently one method widely accepted as being replaceable by an alternative test. Traditionally, agar broth culture and indicator cell

culture have been used to detect mycoplasma contamination. However, the U.S., Japan, and European Union all allow for the substitution of these tests with a PCR-based nucleic acid test [31]. Nucleic acid testing (NAT) has been shown to outperform culture-based methods, in terms of both sensitivity and ability to detect organisms that will not grow in culture [32]. As of 2019, FDA has proposed eliminating the growth-based compendial test for mycoplasma previously outlined, in favor of nucleic acid testing [33]. Originally, manufacturers performed individual comparability studies by showing minimum limits of detection comparable to their own broth-based procedures [34]. An example of this comes from the risk assessment performed for a cell therapy product, which has too short a shelf-life to be tested by conventional methods for mycoplasma [31]. A major support for this shift was facilitated by the creation of international standards by the WHO. In 2015, under the WHO’s auspices, the Mycoplasma Collaborative Study Group performed extensive comparability studies across a number of different mycoplasma species in order to establish minimum limits of detection for nucleic acid-based assays (capable of detecting 10 CFU/mL of *A. laidlawii*) [35]. Establishing this standard facilitated the widespread use of NAT as an alternative. As biomanufacturing expands to include not only proteins but also advanced therapy medicinal products (ATMPs) featuring cells and vectors, establishing global standards for testing like NGS may be a similar necessity. Progress has been made in this regard with the introduction of proposed virus reference standards for adventitious virus detection in NGS to the WHO Expert Committee on Biological Standardization [36].

There is little published literature that directly compares NGS to the *in vivo* adventitious virus test. One approach is to use the existing data comparing the *in vivo* adventitious virus test to the *in vitro* virus test by Gombold [4] and perform NGS experiments on a similar set of viruses as seen in a recent publication [27]. Other fields with concerns about viral contamination, such as agriculture, have also performed comparisons [37]. In these studies, NGS has proven to be more sensitive than existing bioassays, such as symptom development in host indicator plants, and detected previously unknown viruses. Direct comparisons between NGS to classic methods for viral contamination have been performed for specific viruses. Human cytomegalovirus contamination in cell culture was detected by both the *in vitro* virus assay, PCR, and NGS; NGS had a LOD 10 times lower than the *in vitro* virus assay, and directly revealed the contaminating particle’s sequence information [38]. Work is ongoing to address one of the previous weaknesses of NGS, namely the detection of infectious viral particles. NGS has recently been used to investigate active viral infection by using it to view the RNA

transcriptome of viral infection [29,39]. Briefly, all replicating viruses produce RNA transcripts during replication that are not found in non-infectious viral particles. Therefore, detection of such sequences has been reported as a reliable indicator of viral replication using sequencing data alone.

5. Risks of replacing the *in vivo* adventitious virus test with NGS

Replacing a biological adventitious virus test with a nucleic acid-based method, such as NGS, is not perceived to come without risk. Broadly, those risks can fall into four distinct categories: risk to the product, technical risk, regulatory risk, and business risk. The following discussion will look at each of these risks in turn to aid in the decision-making of biopharmaceutical manufacturers.

Risks to the product and technical risk are closely related; therefore, they are considered together. These risks can include:

1. False negatives, where a potential viral contaminant is not detected;
2. Ambiguity in the final result (e.g., difficulty in distinguishing between true positive and true negative, detection of unknown sequences, challenge and complexity of the bioinformatic analysis);
3. Variability of databases, and their upkeep.

Bullet points 1 and 2 relate to the false positive and false negative rate of NGS. While biopharmaceutical manufacturer experience with NGS is significantly less than that of the *in vivo* adventitious virus test and the *in vitro* virus test, the CAACB collected data on false positives and false negatives experienced by member companies when using NGS. Of the data reported to the CAACB, there was only one case of an NGS false positive and zero cases of NGS false negatives. In the reported case, the false positive was attributed to likely cross contamination during sample handling, though it is important to note that this false positive experience did not occur in a GMP setting. As discussed earlier, both false positives and false negatives have occurred with the *in vivo* adventitious virus test.

While ambiguity of the final assay result is a risk, it is not limited to only NGS. All assays suffer, to some degree, from difficulty in distinguishing between true positives and true negatives. *In vivo* assays, for example, are based on observing animal health or cell cytopathology, which may not always be straightforward, for example interpreting a specific animal behavior as due to a viral infection. Broad agreement on what constitutes a true positive NGS result would be helpful to the biopharmaceutical and viral safety community.

Unassigned or unknown sequences are often considered a risk of using NGS as a tool for viral safety; however, it is important to take the possibility of such sequences in context and consider both the intended use of the assay and a risk assessment. CAACB members were asked if they have ever observed a nucleic acid sequence that was not able to be classified by their bioinformatic algorithm and database. Of those that perform NGS, 50% indicated that they have not observed nucleic acid sequences that were not able to be classified and 50% have. However, characteristics of the reads, such as depth, quality, strand polarity, length of the read, contig match and assembly into a larger contig, as well as strength of the assignment can inform whether additional follow up may be necessary. Additionally, it was reported that some sequencing pipelines intentionally do not attempt to classify some reads, such as reads that are not classified as viral.

NGS approaches may be developed on a per-company basis, either in-house or at contract testing labs. These approaches may, therefore, use proprietary bioinformatic pipelines or customized databases. Further, the database will be part of the bioinformatics pipeline validation. Therefore, any change to the database, i.e., to ensure it remains current, must be properly documented and tests must be in place to ensure that the change in the database will still allow the detection of viruses in predefined test cases. Only when a database has passed these tests, will it be useable in a GMP pipeline. The aforementioned

variability, together with challenges in keeping databases current, may be a risk to maintaining GMP compliance.

Risks related to regulatory authorities include: delayed regulatory approval due to implementation of a new method or replacement of an old method; the risk that regulatory authorities will not accept the NGS validation approach due to lack of clarity on how NGS should be validated. Both risks reflect the general lack of experience of NGS in applications and for regulators to review and respond to those applications. Until more experience can be gained, generation of robust data and engagement with regulators early and often is the most likely method to mitigate this risk. In the U.S. and EU, there are several methods through which companies can interact with regulators to gain feedback on their proposed approach. Further, there are industry-led initiatives that will look to provide guidance on how companies should approach validation of an NGS method. This will continue to be a risk until these methods are considered current. Efforts are currently ongoing to address this need, such as the collaborative studies performed by PDA AVDTIG.

Additionally, some companies may choose to do NGS concurrently, as a supplement, with the *in vivo* adventitious virus test. This will increase costs and has the potential to delay product development, clinical timelines, or product release due to false-positive and follow-up investigation.

Finally, there is both regulatory and business risk in the current lack of harmonization across regulatory authorities in their acceptance of NGS as an alternative to the *in vivo* adventitious virus test. In the worst-case scenario, this risk could lead to a similar outcome as noted earlier where both the new and existing methodologies may need to be performed for some regulators until sufficient data can be collected to adequately demonstrate that NGS could replace an existing biological method.

6. Challenges to implementing NGS

In addition to the risks noted above, implementing NGS within a biopharmaceutical manufacturing organization for virus safety carries several challenges. Conceptually, these can be thought of as falling into technical challenges, regulatory challenges, and business challenges; however, it is important to note that regulatory and business challenges largely stem from continuing technical challenges.

Technical challenges include, but are not limited to, the following:

1. Sample isolation and handling
 - a. Development of a validated step to capture different viral genomes (e.g., single stranded, double stranded, RNA, and DNA) and types in the context of overwhelming amounts of cellular DNA and RNA;
 - b. Maintaining sample integrity during transport, storage, and processing to reduce degradation, especially RNA genomes;
2. Bioinformatics
 - a. Lack of in-house expertise to use NGS bioinformatic tools and analyze NGS data;
 - b. NGS data and bioinformatic tools used for analysis require investments in infrastructure as well as specialized knowledge and skills;
 - c. Lack of guidance on setting thresholds used by bioinformatic algorithms;
3. Standards - lack of standards and reference materials to support comparability testing;
4. Current state of the technology - the length of NGS analysis time may still be too long for use with some product modalities.

The first two items are related to in-house expertise for sample preparation and nucleic acid extraction. There is collaborative work on sample preparation that has been published by the PDA AVDTIG [16]. However, developing such in-house experience and expertise will

require dedicated resources.

In the case of in-house expertise and the ease of use of bioinformatic tools, there are a growing number of bioinformatic degree-granting programs that may eventually fill this gap. Until such time as the supply of bioinformatic experts meets the demand, biopharmaceutical development and manufacturing may need to rely on outside testing labs to perform NGS or bioinformatic analysis.

The lack of standards and reference materials for use in NGS comparability studies is a well-known problem and is currently being addressed through a number of different forums. Examples include the development of reference material for adventitious virus detection by NIBSC in the UK [40], the development of well characterized viral stocks as a part of the PDA AVDTIG effort that have also been presented to the WHO Expert Committee on Biological Standardization as reference standards for adventitious virus detection [36], and recent NIST and FDA workshops on viral standards for NGS [41].

The duration of NGS analysis may still be too long for use with product modalities that have short shelf lives (e.g., <1 week); however, all broad-spectrum adventitious virus detection technologies are too lengthy. As such, there is an opportunity for additional technology development to shorten assay times or advance novel approaches beyond the current NGS approach.

Regulatory challenges to implementing NGS are largely the same as those mentioned above, with the largest challenges currently being the lack of familiarity or comfort with NGS as a viral safety tool. Additionally, the lack of a harmonized regulatory framework with regards to test design, acceptance criteria, databases, and validation approach continues to be a challenge. These challenges are also mirrored in a 2019 Biophorum Operations Group (BPOG) report on rapid microbiological methods which listed the top three industry concerns as: regulatory acceptance of the new method, filing a regulatory change control, and time to validate a non-compendial method [42].

In addition to the risks noted above, the business challenges to implementing NGS include gaining buy-in from upper management to invest resources in infrastructure and method development without a guaranteed regulatory approval. Method development may be especially challenging as aspects of the current bioinformatic pipelines may be proprietary, meaning that there are fewer than expected generalizable tools available for use off the shelf.

7. Suggestions and recommendations

In conclusion, as presented above, of the three reported *in vivo* adventitious virus test true positive events, all were also detected in another concurrent assay (*in vitro*). Current adventitious agent assays, including the *in vivo* adventitious virus test, have missed viral contaminants in biopharmaceutical products [1,2] and cell lines [19], providing support that the breadth of detection is actually broader with NGS, ensuring greater product safety. Further, the sensitivity of the *in vivo* adventitious virus test is generally less than other assays [4,27]. Collectively, this indicates the potential for improvement over the *in vivo* adventitious virus test as a current standard method for viral safety assurance. This is especially true when an alternative method, with equivalent or greater breadth of detection and sensitivity, exists.

Therefore, it is proposed to update the overall virus safety program for new biopharmaceutical products through replacing the *in vivo* adventitious virus testing approaches with modern methodologies that maintain or even improve the final safety of the product.

While the replacement of the *in vivo* adventitious virus test with NGS comes with several risks and challenges, as discussed above, it is important to view those challenges considering the benefits.

- It is broad spectrum, can be operated in an agnostic manner, and has detected previously undetected viral contaminants in a biopharmaceutical context [2,19].

- It has comparable or better sensitivity than the *in vitro* virus test and the *in vivo* adventitious virus test (when compared to viruses previously tested *in vivo*) [27].
- It simultaneously detects and identifies the contaminant.
- Some applications of NGS have been reported to take less time than the *in vivo* adventitious virus test and the *in vitro* virus test.

Therefore, the replacement of *in vivo* adventitious virus testing with NGS, together with *in vitro* virus testing, could comprise a comprehensive adventitious virus detection program.

The greatest perceived risk and challenge to replacing an existing assay with NGS is related to the lack of regulatory acceptance and harmonization. Therefore, it is recommended that where possible, organizations conduct regular meetings with regulatory authorities, such as Type C meetings or discussions and presentations to the FDA Emerging Technology Team in the U.S., to gain feedback throughout the development and validation of the new method. These meetings can ensure that key questions are addressed early and that regulators are familiar and comfortable with the approach. Publication of data demonstrating the capabilities of NGS, revision of guidance documents, as exemplified in the current revision of ICH Q5A, and future collaborative efforts will be necessary to enable broad exposure and ensure that NGS and the assay it is replacing are not required to be implemented concurrently.

Where the biopharmaceutical manufacturing industry is likely to meet the least resistance in implementing new adventitious virus detection technologies is when traditional testing methods are not suitable for use with a particular modality for the development and approval of a product. Examples of these types of cases include live viral vaccines for which the development of a neutralizing antibody is not possible, cellular therapies, and treatments for emergent health crises as seen in the COVID-19 global pandemic. Therefore, it is recommended that any new product modality that is based on or uses mammalian cell banks (e.g., genetically modified cells for viral vector production, allogeneic cell therapies, iPSC derived products, exosomes, etc.) use NGS at the earliest stage of process development to mitigate the safety, regulatory, and business risk for the future.

Continued collaboration across industry will allow the generation and publication of data to further support the suitability of NGS as a viral safety method, familiarize both regulators and industry on the capabilities and limitations of the technique, and provide guidance on structured approaches to qualification and validation of an NGS methodology.

Finally, it is important to remember that testing is only one component of a company's larger viral safety control strategy and the replacement of a specific viral safety assay with another should be considered through the lens of an overall holistic end-to-end risk assessment. Ideally, the data presented here, in conjunction with in-house data, will aid in that process and quantify the risk of removal and replacement of one specific assay.

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