Sensitivity and breadth of detection of high-throughput sequencing for adventitious virus detection

High-throughput sequencing (HTS) is capable of broad virus detection encompassing both known and unknown adventitious viruses in a variety of sample matrices. We describe the development of a general-purpose HTS-based method for the detection of adventitious viruses. Performance was evaluated using 16 viruses equivalent to well-characterized National Institutes of Health (NIH) virus stocks and another six viruses of interest. A viral vaccine crude harvest and a cell substrate matrix were spiked with 22 viruses. Specificity was demonstrated for all 22 viruses at the species level. Our method was capable of detecting and identifying adventitious viruses spiked at $10^4$ genome copies per milliliter in a viral vaccine crude harvest and 0.01 viral genome copies spiked per cell in a cell substrate matrix. Moreover, 9 of the 11 NIH model viruses with published in vivo data were detected by HTS with an equivalent or better sensitivity (in a viral vaccine crude harvest). Our general-purpose HTS method is unbiased and highly sensitive for the detection of adventitious viruses, and has a large breadth of detection, which may obviate the need to perform in vivo testing.

npj Vaccines (2020) 5:61; https://doi.org/10.1038/s41541-020-0207-4

INTRODUCTION

The potential contamination of biopharmaceuticals with adventitious (unintentionally introduced) viruses poses a serious safety risk and threatens public confidence in the use of biopharmaceuticals. This is particularly true in the case of vaccines administered to large numbers of healthy people, including children. There have been a number of instances over previous decades where evidence for the presence of adventitious virus contamination in a marketed vaccine product has threatened public trust in immunization programs. Examples include the detection of simian virus 40 (SV40) in the early polio vaccine in the 1960s, the finding of reverse transcriptase in measles and mumps vaccines in 1995 and of porcine circovirus (PCV) nucleic acid sequences and/or infectious circovirus in rotavirus vaccines in 2010.

These viruses could be unintentionally introduced at various manufacturing stages and may originate from multiple sources including raw materials, the cell substrate or the environment. Implementation of Good Manufacturing Practise and close monitoring of the manufacturing process can help reduce the likelihood of viral contamination. Therefore, adventitious virus testing at various stages of the manufacturing process is an integral part of the safety assessment for vaccines and other biological products, and there are well-defined regulatory requirements to ensure that these products are absent of adventitious viruses.

Gaps exist in the current compendial adventitious virus testing package where some viral families are not detected or incompletely detected by the compendial methods. Non-specific adventitious virus testing of biological materials has typically included in vivo tests and cell culture-based in vitro tests, the breadth and sensitivity of which are presumed from historical experience rather than from systematic assay validation. In addition, routine testing for adventitious viruses following the compendial requirements generally requires testing the cell substrate and the viral crude harvest (at seed lot and/or bulk levels). Both of these manufacturing stages present complex matrices for in vivo and in vitro testing. Targeted PCR-based virus detection methods require prior knowledge of the adventitious virus with respect to primer design/selection to target specific nucleic acid sequences. As such, there is a need for a broad-range detection method for both anticipated viruses, as well as unanticipated viruses.

A study by the National Institutes of Health (NIH) in 2014 compared the sensitivity of in vivo assays and in vitro cell culture tests using a panel of 16 viruses and found the in vitro tests to be more sensitive for detecting most of the tested viruses. The results from the NIH study support the use of tests for broader detection of adventitious viruses, particularly where no suitable animal models or appropriate culture methods for detection exist. The World Health Organization (WHO; WHO technical report series 978 [Annex 3]) and the European Pharmacopoeia (legally binding standards and quality specifications; Ph. Eur. 2.6.16, Ph. Eur. 5.2.14, Ph. Eur. 5.2.3) recommend or require that prior to the implementation of new alternative methods to detect adventitious agents, the specificity and sensitivity of the new and existing methods must be compared; and whether the new method has at least the same sensitivity as in vivo methods should be determined (Supplementary Table 1).

High-throughput sequencing (HTS) is a non-specific technique with the potential to detect both known and unknown adventitious agents including viruses. High-throughput molecular biology methods (HTS combined with a pan-viral microarray) had succeeded in detecting the contamination of Rotarix vaccine by a porcine circovirus. HTS may also be more sensitive than quantitative PCR (qPCR); however the method may be overly sensitive to the detection of background and cross-contaminating viral nucleic acids originating from the laboratory environment or from other sources. Viral genome size can also influence the sensitivity of HTS as sensitivity is expected to be proportional to the mass ratio of nucleic acids in a given matrix. Although HTS results for adventitious virus testing may differ between laboratories, the development of well-
characterized model virus stocks would support standardization and validation of the different HTS platforms. Currently there are no established viral reference standards with corresponding in vivo data, for assessing new techniques for the detection of adventitious viruses. The panel of 16 virus stocks from the NIH is instrumental in our efforts to develop, qualify and validate the HTS adventitious virus test, by providing an important baseline against which new techniques for the detection of adventitious viruses can be compared. Here, we describe the development of a general-purpose HTS-based test for the detection of adventitious viruses and its performance using virus stocks equivalent to the 16 NIH model viruses, plus another six viruses of interest. Detection of these viruses was assessed in a live Yellow Fever virus vaccine crude harvest matrix and a Vero cell substrate matrix to define the sensitivity (limit of detection [LOD]) and to demonstrate the specificity of the HTS test for adventitious virus testing.

## RESULTS

Identification of approximate LOD and viral spike detection in a viral vaccine crude harvest nucleic acid extraction

Nucleic acid recovery for both the Yellow Fever Virus vaccine crude harvest and the Vero cell substrate matrix are shown in Supplementary Note 1.

The number of sequence reads detected for each of the spiked viruses is listed in Table 1. All 22 model adventitious viruses were detected in at least one of the spike levels assessed. LOD was determined to be approximately $10^3$ to $10^4$ genome copies per mL.

### Table 1. Number of sequence reads detected for each of the spiked viruses at the different spiking levels in the viral vaccine crude harvest.

| Virus                     | Spiking level (GC mL$^{-1}$) | $10^5$ | $10^4$ | $10^3$ | $10^2$ | $10^1$ |
|---------------------------|-----------------------------|-------|-------|-------|-------|-------|
| NIH                       | Total raw reads              | 291,341,932 | 371,184,528 | 365,131,932 | 333,373,964 | 437,070,286 | 332,181,730 |
| Adenoviridae              | Adenovirus 5                 | 14,382 | 2284  | 264   | 193   | 34    |
| Adenovirus 41             | 1154                        | 164   | 12    | 16    | 11    | 6     |
| Flaviviridae              | Bovine Viral Diarrhea Virus  | 95,666 | 6306  | 776   | 926   | 68    |
| Herpesviridae             | Herpes Simplex Virus Type 1  | 1366  | 200   | 40    | 23    | 6     |
| Simian Cytomegalovirus    | 49,266                      | 9386  | 1170  | 676   | 116   |
| Orthomyxoviridae          | Influenza A Virus            | 16,553 | 1266  | 83    | 166   | 8     |
| Paromyxoviridae           | Bovine Parainfluenza Virus Type 3 | 48,578 | 3548  | 321   | 410   | 24    |
| Measles Morbillivirus     | 12,571                      | 922   | 87    | 105   | 10    |
| Mumps Virus               | 9041                        | 524   | 86    | 46    | 10    |
| Picornaviridae            | Coxsackievirus A16          | 351   | 27    | 1     | 0     | 3*    |
| Coxsackievirus B3         | 2036                        | 157   | 10    | 18    |
| Echovirus 11              | 2539                        | 140   | 4     | 14    | 5*    | 1     |
| Rhinovirus 2              | 1259                        | 62    | 6     | 20    | 0     |
| Polymaviridae             | Simian Virus 40             | 528   | 88    | 47    | 15    | 10    |
| Rhabdoviridae             | Vesicular Stomatitis Virus  | 13,120 | 1071  | 102   | 157   | 27    | 12    |
| Togaviridae               | Rubella Virus               | 4610  | 391   | 76    | 51    | 4     |
| SP                        | Bornaviridae                | 341   | 6     | 1     | 0     | 0     |
| Coronaviridae             | Bovine Coronavirus          | 7447  | 473   | 48    | 46    | 5*    | 2     |
| Herpesviridae             | Human Cytomegalovirus       | 162,008 | 28,013 | 2733  | 2657  | 305   | 252   |
| Paroviridae               | Minute Virus of Mice        | 319   | 48    | 12    | 10    | 0     |
| Porcine Parvovirus        | 146,720                     | 20,668 | 2412  | 2609  | 397   | 238   |
| Reoviridae                | Reovirus Type 3             | 16    | 0     | 0     | 0     | 0     |

Replicate spikes were made at $10^3$ and $10^2$ GC/mL as it was hypothesized that the LOD may be near this spike level. GC genome copy, NIH National Institutes of Health, SP Sano fi Pasteur. *3 or more reads belonging to one contig that is less than 201 bp (does not meet criteria for positive result).
of Yellow Fever Virus vaccine crude harvest. Specificity was assessed for all 22 viruses by determining whether the bioinformatics tool PhyloID was able to unequivocally detect each of the 22 spiked-in viruses and that none of these viruses was detected in the negative control (unspiked Yellow Fever virus vaccine crude harvest). Specificity was verified for all 22 viruses at the species level while specificity was verified for 20 of the 22 viruses at the strain level. For mammalian reovirus type 3 and coxsackievirus B3, the reads from the virus used for the spike were spread over two (for coxsackievirus B3) or three (for mammalian reovirus type 3) reference sequences of the correct viral species.

**Table 2.** Repeatability of the number of reads detected by the HTS test across 3 replicate datasets in the viral vaccine crude harvest.

| Spiked virus                      | 10^4 GC mL⁻¹ replicate 1 | 10^4 GC mL⁻¹ replicate 2 | 10^4 GC mL⁻¹ replicate 3 | Mean number of reads at 10^4 genome copies per mL | Standard deviation | % Coefficient of variation |
|-----------------------------------|---------------------------|--------------------------|--------------------------|---------------------------------------------------|-------------------|---------------------------|
| NIH Adenoviridae                  |                           |                          |                          |                                                   |                   |                           |
| Adenovirus 5                      | 2284                      | 1792                     | 2570                     | 2215                                              | 394               | 18                        |
| Adenovirus 41                     | 164                       | 120                      | 176                      | 153                                               | 29                | 19                        |
| Flaviviridae                      |                           |                          |                          |                                                   |                   |                           |
| Bovine Viral Diarrhea Virus       | 6306                      | 18,680                   | 21,484                   | 15,490                                            | 8076              | 52                        |
| Herpesvirida                      |                           |                          |                          |                                                   |                   |                           |
| Herpes Simplex Virus Type 1       | 200                       | 255                      | 309                      | 293                                               | 33                | 11                        |
| Simian Cytomegalovirus            | 9386                      | 3,760                    | 5,080                    | 6,075                                             | 2942              | 48                        |
| Orthomyxovirida                   |                           |                          |                          |                                                   |                   |                           |
| Influenza A Virus                 | 1266                      | 21                       | 33                       | 5,408                                             | 3953              | 73                        |
| Paramyxovirida                    |                           |                          |                          |                                                   |                   |                           |
| Bovine Parainfluenza Virus Type 3 | 3548                      | 8760                     | 10,795                   | 7701                                              | 3738              | 49                        |
| Measles Morbillivirus             | 922                       | 1950                     | 1778                     | 1550                                              | 551               | 36                        |
| Mumps Virus                       | 524                       | 3602                     | 5741                     | 3289                                              | 2623              | 80                        |
| Picornavirida                     |                           |                          |                          |                                                   |                   |                           |
| Coxsackievirus A16               | 27                        | 112                      | 53                       | 64                                                | 44                | 69                        |
| Coxsackievirus B3                 | 157                       | 969                      | 986                      | 704                                               | 474               | 67                        |
| Echovirus 11                      | 140                       | 847                      | 1,854                    | 947                                               | 861               | 91                        |
| Rhinovirus 2                      | 62                        | 238                      | 197                      | 166                                               | 92                | 55                        |
| Polyomavirida                     |                           |                          |                          |                                                   |                   |                           |
| Simian Virus 40                   | 88                        | 62                       | 75                       | 75                                                | 13                | 17                        |
| Rabdovirida                       |                           |                          |                          |                                                   |                   |                           |
| Vesicular Stomatitis Virus        | 1071                      | 9257                     | 13,396                   | 7908                                              | 6272              | 79                        |
| Togavirida                        |                           |                          |                          |                                                   |                   |                           |
| Rubella Virus                     | 391                       | 560                      | 796                      | 582                                               | 203               | 35                        |
| NIH National Institutes of Health, SP Sano Pasteur. |
| Coronavirida                      |                           |                          |                          |                                                   |                   |                           |
| Bovine Coronavirus                | 473                       | 1369                     | 1813                     | 1218                                              | 683               | 56                        |
| Herpesvirida                      |                           |                          |                          |                                                   |                   |                           |
| Human Cytomegalovirus             | 28,013                    | 139,737                  | 66,743                   | 162,203                                            | 108,453           | 67                        |
| Parovirida                        |                           |                          |                          |                                                   |                   |                           |
| Minute Virus of Mice              | 104                       | 130                      | 1813                     | 94                                                | 42                | 45                        |
| Porcine Parovirus                 | 20,668                    | 54,941                   | 15,775                   | 30,461                                             | 21,341            | 70                        |
| Reovirida                         |                           |                          |                          |                                                   |                   |                           |
| Reovirus Type 3                   | 0                         | 120                      | 49                       | 56                                                | 60                | 107                       |

Assessment of LOD
Using a range of viral spikes from 10^5 to 10^3 genome copies per mL, most viruses were consistently detected at least at 10^4 genome copies per mL in viral vaccine crude harvest (Table 2). Reproducibility of the number of reads detected by the HTS test across three replicate datasets in the viral vaccine crude harvest

Reproducibility of the test was evaluated using three independent replicates (over two spiking studies) at a spiking level of 10^4 genome copies per mL. The mean number of reads at 10^4 genome copies per mL is shown in Table 2; the coefficient of variation ranged from 11 to 107%.

Identification of approximate LOD and viral spike behavior in the cell substrate matrix
The number of sequence reads detected for each of the spiked viruses is listed in Table 3. For the Vero cell substrate with a cell count of 1 × 10^6 cells per mL, 19 of the 22 viruses were detected at least at 10^4 genome copies per mL (0.01 genome copies per cell) in the cell substrate matrix, with potentially better sensitivity for some viruses. Influenza A virus, minute virus of mice and reovirus

Table 2. Repeatability of the number of reads detected by the HTS test across 3 replicate datasets in the viral vaccine crude harvest.
19 of the 22 viruses were detectable at detection that is at or below 0.01 genome copies per cell in a Vero cell substrate matrix (Table 3). 3 were not detected at this highest spike level of 0.01 genome copies per cell.

**LOD overview**

Preliminary evaluation indicates that the adventitious virus detection test using HTS can potentially achieve a limit of detection that is at or below 0.01 genome copies per cell in a Vero cell substrate matrix (Table 3). 19 of the 22 viruses were detectable at 0.01 genome copies per cell and 14 of the viruses were detected at 0.001 genome copies per cell in a Vero cell substrate matrix (Table 3).

In the Yellow Fever Virus vaccine crude harvest, HTS can achieve a LOD that is at or below $10^4$ genome copies per mL. Of the viruses assessed, 21 were detectable at $10^4$ genome copies per mL, with 16 of these at $10^3$ genome copies per mL in a matrix containing $10^9$ viral vaccine genome copies per mL (Fig. 1).

**Comparison to NIH in vivo testing results**

In the previous NIH study, 11 of the 16 virus stocks were assessed for their detection in the in vivo test. While the in vivo assays detect infectious virus, with levels reported as titer, the HTS method measures nucleic acids. We converted the sensitivity reported in the NIH study into genome copies using the infectious titer to genome copy ratio determined from the vial of original NIH viral stock. Nine of the 11 viruses previously assessed in vivo were detected with better sensitivity by our HTS method in the viral vaccine crude harvest matrix than reported in vivo in the NIH study; the remaining five viruses were not assessed in vivo within the NIH study (Table 4). Influenza A virus and VSV were detected with better sensitivity in vivo in the NIH study than using HTS in this study; these viruses were also detected at $10^4$-fold and $10^5$-fold lower genomic copy amounts in vivo compared to in vitro, respectively, as the animal model allows an efficient amplification of these replicating viral contaminants.

**DISCUSSION**

Various reviews of HTS for adventitious agent detection have shown that there is a need for unbiased extraction methods, relevant controls, the use of spike recovery experiments, and quality control measures during library preparation. Our HTS method is a general-purpose non-specific method that can be used to detect both known and unexpected viruses in multiple types of samples (viral seeds/crude harvest, cell banks, and cell supernatants). The LOD for a panel of 22 model adventitious viruses was assessed in a viral vaccine crude harvest matrix and a cell substrate matrix. Based on the results, the adventitious virus test using HTS technology is capable of detecting adventitious viruses at least at $10^4$ genome copies per mL in the viral vaccine crude harvest matrix. Detection in the cell substrate matrix was at least at 0.01 viral genome copies per cell ($10^4$ genome copies per mL) for 19 of the 22 viruses. In a similar study evaluating the performance of HTS for the detection of adventitious viruses by spiking model viruses into a cellular matrix containing whole cells, all of the model viruses used (human respiratory syncytial virus [RSV], Epstein-Barr virus [EBV], feline leukemia virus and human reovirus) were detected when spiked at 3 genome copies per cell; only EBV and RSV were detected at 0.1 genome copies per cell. This study showed that HTS can detect almost all viral contaminants evidenced by the large panel that was investigated, i.e., 22 different viruses that were enveloped/non-enveloped, single-stranded/double-stranded, RNA, DNA and of varying genome sizes. The use of the method for different sample types was also demonstrated, i.e., the nucleic acid recovery from the viral vaccine crude harvest matrix was higher than the cell substrate matrix, however a high proportion of contaminants was detected for both samples. The difference in detection levels observed for some of the viruses is likely attributed to a higher amount of background host nucleic acids from the cell substrate. Compared to the vaccine viral harvest, where even if the cells are lysed during manufacturing, the host cell nucleic acids are limited within the viral harvest, which is very different when testing the cell substrate matrix. These cells are lysed during sample preparation, releasing their nucleic acids, and will therefore make up a higher proportion of the sequence data leading to a poorer signal (viral spike) to noise ratio and a decrease in the number of reads detected for the viral spikes. Overall, the successful detection of the spiked-in viruses in these complex backgrounds demonstrates the applicability of HTS for adventitious virus detection.

---

**Table 3.** Number of sequence reads detected for each of the spiked viruses at the different spiking levels in the cell substrate matrix.

| Virus | Genome copies per cell |
|-------|------------------------|
|       | 0.01 | 0.01 | 0.001 | 0.001 |
| NIH   |       |       |       |       |
| Adenoviridae |       |       |       |       |
| Adenovirus 5  | 244  | 125  | 18   | 4    |
| Adenovirus 41 | 21   | 8    | 0    | 0    |
| Flaviviridae |       |       |       |       |
| Bovine Viral Diarrhea Virus | 1014 | 1096 | 11   | 12   |
| Herpesviridae |       |       |       |       |
| Herpes Simplex Virus Type 1 | 58   | 17   | 0    | 0    |
| Simian Cytomegalovirus | 449  | 386  | 2    | 32   |
| Orthomyxoviridae |       |       |       |       |
| Influenza A Virus | 0*   | 0*   | 0*   | 0*   |
| Paramyxoviridae |       |       |       |       |
| Bovine Parainfluenza Virus Type 3 | 627  | 512  | 25   | 11   |
| Measles Morbillivirus | 43   | 45   | 0    | 0    |
| Mumps Virus | 181  | 253  | 2    | 4    |
| Picornaviridae |       |       |       |       |
| Coxsackievirus A16 | 10   | 9    | 0    | 0    |
| Coxsackievirus B3 | 55   | 106  | 0    | 0    |
| Echovirus 11 | 110  | 86   | 0    | 0    |
| Rhinovirus 2 | 6    | 20   | 0    | 0    |
| Polyomaviridae |       |       |       |       |
| Simian Virus 40 | 48   | 29   | 1    | 0    |
| Rhabdoviridae |       |       |       |       |
| Vesicular Stomatitis Virus | 580  | 674  | 5    | 7    |
| Rubella Virus | 130  | 41   | 0    | 0    |
| SP     |       |       |       |       |
| Bornaviridae |       |       |       |       |
| Human Borna Disease Virus | 14   | 10   | 0    | 9    |
| Coronaviridae |       |       |       |       |
| Bovine Coronavirus | 35   | 41   | 0    | 2    |
| Herpesviridae |       |       |       |       |
| Human Cytomegalovirus | 12,990 | 13,089 | 554 | 551 |
| Paroviridae |       |       |       |       |
| Minute Virus of Mice | 0*   | 14*  | 0*   | 0*   |
| Porcine Parovirus | 5428 | 4932 | 327  | 214  |
| Reoviridae |       |       |       |       |
| Reovirus Type 3 | 0*   | 0*   | 0*   | 0*   |

Replicate spikes were made at each spiking level. GC genome copy. *Indicates that the LOD for the specific virus was not identified through this study.
Methods for the detection of adventitious agents continue to evolve and improve. The use of viral stocks equivalent to the well-characterized viral stocks from the NIH-supported studies allows us to compare our HTS performance against compendial in vivo testing, as proposed in the European Pharmacopoeia Chapter 5.2.14. In addition, such well-characterized materials can be used as a reference to help ensure satisfactory test performance in many matrices and thus, enable more meaningful comparisons between laboratories. Importantly, the use of HTS is in-line with the Directive 2010/63/EU of the European Parliament and of the Council of 22 September 2010 with the aim, and the need, to reduce the number of animals used for scientific purposes.

The approach to this study is based on European Pharmacopoeia chapter 5.2.14 (Supplementary Table 1) and uses 16 well-characterized model viruses that are representative of potential contaminants in vaccine manufacturing to demonstrate that HTS sensitivity is at least equivalent to the sensitivity of the in vivo test methods. In this study, we produced 16 viruses that were equivalent to the viruses assessed in the NIH study and 9 of our 11 viruses were detected with better sensitivity by HTS in the viral vaccine crude harvest compared with the NIH in vivo data. Influenza A virus and VSV were the two viruses detected with lower sensitivity by HTS (also detected with lower sensitivity in vivo) which most likely suggests that the animal models used provide a good environment for the replication of those viruses, while HTS does not involve any viral replication. Two viruses resolved to the species level, but not to the strain level (mammalian reovirus type 3 and coxsackievirus B3) when measured by the in-house Sanofi Pasteur tool PhyloID. However, resolution at the strain level can be more difficult when counts are low and when strains are similar in their sequences, as the reads may get assigned to any of the reference genomes within that species. We are, however, working on improving the strain resolution of PhyloID.

A limitation of a HTS adventitious virus detection method is that the test only detects viral nucleic acid and further investigation is necessary to determine if the signal is from infectious particles. In some cases, for example gamma-irradiated raw materials, or inactivated samples, the presence of viral nucleic acids is not necessarily a concern. In addition, HTS may be prone to detection of background and cross-contaminating viral nucleic acids originating from the laboratory environment or from other sources due to its breadth of detection, as well as its sensitivity. A thorough understanding of the testing environment, proper test controls and knowledge of the history of the test sample will help to determine if laboratory follow-up is necessary.

Possible laboratory follow-ups include confirmation of intact full-length viral genomes, expression analysis, and infectivity assays. It is also essential to validate the HTS test under Good Manufacturing Practice in order to apply the method to new vaccine products as release specification tests, as a substitution of in vivo tests to detect adventitious virus and to potentially replace/substitute specific tests such as PCRs used in routine testing. HTS would need to be compared with qPCR if it is to become routinely used. The sensitivity of HTS is influenced by genomic size, as well as genomic structure, and for RNA viruses, the relative efficiencies in reverse transcription and cDNA synthesis. Nevertheless, HTS is virus-sequence independent and can detect all types of virus genomes, including single-stranded/double-stranded RNA and DNA.

The HTS method for adventitious virus detection is a highly sensitive, unbiased non-specific method, which has a large breadth of detection compared to other tests, a favorable comparison to in vivo data and may obviate the need to perform in vivo testing for adventitious viruses.
METHODS

A schematic overview of the HTS adventitious virus detection procedure is provided in Fig. 2.

Preparation of virus panel

Two vials of each of the 16 virus stocks were received from the NIH; the viruses are described in Supplementary Table 2. The genome copy (GC) of each stock was determined by qPCR by Clean Cells (https://clean-cells).

Table 4. Comparison of the sensitivity in a viral vaccine crude harvest versus sensitivity of the NIH in vivo tests.

| Virus Family     | NIH virus | In vivo LOD (TCID<sub>50</sub>, as described previously)<sup>a</sup> | Sensitivity of the in vivo assay (GC, determined by Clean Cell) | Sensitivity of the HTS assay (GC) |
|------------------|-----------|---------------------------------------------------------------|-----------------------------------------------------------------|----------------------------------|
| Adenoviridae     | Adenovirus 5 | NT                                                             | NT                                                              | 90                               |
|                  | Adenovirus 41 | NT                                                             | NT                                                              | 9000                             |
| Flaviviridae     | Bovine Viral Diarrhea Virus | Not detected                                                  | ≥3.39 × 10<sup>8</sup>                                             | 90                               |
| Herpesviridae    | Herpes Simplex Virus Type 1 | 100                                                            | 3.4 × 10<sup>4</sup>                                             | 900                             |
|                  | Simian Cytomegalovirus | NT                                                            | NT                                                              | 90                               |
| Orthomyxoviridae | Influenza A Virus | 10<sup>-4</sup>                                               | 5.46 × 10<sup>-4</sup>                                           | 90<sup>*</sup>                  |
| Paramyxoviridae  | Bovine Parainfluenza Virus Type 3 | Not detected                                                  | ≥8.67 × 10<sup>2</sup>                                           | 90                               |
|                  | Mumps Virus | Undiluted                                                     | 1.12 × 10<sup>9</sup>                                           | 900                             |
|                  | Measles Morbillivirus | Not detected                                                 | ≥4.00 × 10<sup>8</sup>                                           | 900                             |
| Picornaviridae   | Coxsackievirus A16 | 10<sup>5</sup>                                                | 3.03 × 10<sup>9</sup>                                           | 9000                            |
|                  | Coxsackievirus B3 | 1                                                             | 3.0 × 10<sup>9</sup>                                           | 900                             |
|                  | Echovirus 11 | Not detected                                                  | ≥6.29 × 10<sup>9</sup>                                           | 900                             |
|                  | Rhinovirus 2 | NT                                                            | NT                                                              | 9000                            |
| Polyomaviridae   | Simian Virus 40 | NT                                                            | NT                                                              | 9000                            |
| Rhabdoviridae    | Vesicular Stomatitis Virus | 10<sup>-4</sup>                                               | 1.28 × 10<sup>-4</sup>                                           | 90<sup>*</sup>                  |
| Togaviridae      | Rubella Virus | Not detected                                                  | ≥2.45 × 10<sup>7</sup>                                           | 900                             |

HTS assay is corrected for the volume used for extraction (900 μL).

GC genome copy, HTS high-throughput sequencing, ND Not detected, NIH National Institutes of Health, NT Not tested.

*viruses with lower sensitivity using the HTS test compared to the in vivo assay.

Fig. 2  Overview of HTS for adventitious virus detection. Extraction of the sample in order to recover all types of nucleic acid, followed by second-strand synthesis and sequencing library preparation. Data analysis was carried out by PhyloID (a Sanofi Pasteur developed analysis pipeline). ds double-stranded, ss single-stranded.

Fig. 3  General overview of viral nucleic acid extraction protocol. cDNA complementary DNA, ds double-stranded, PCR polymerase chain reaction.
Limit of detection (LOD)

The limit of detection of adventitious viruses using HTS was compared to the data obtained from the NIH study. The LOD achieved by the in vivo test methods, as described in the NIH study, was converted to genome copies based on a determination of genome content of the NIH stocks by Clean Cells to facilitate the comparison with the HTS studies described here.

The genome copies at LOD for the in vivo test were calculated using the formula:

\[
\frac{\text{Stock infectious titer}}{10^9 \text{PFU} \times (3.23 \times 10^5 \text{ genome copies per mL})} = 34,000 \text{ genome copies}
\]

*All obtained from the NIH stock, †As determined by Clean Cells from the NIH stock.

(The limit of detection for viruses not detected in vivo is specified as greater than the genome copy number after conversion from the titer of the NIH stock virus [Supplementary Tables 2 and 3]).

DATA AVAILABILITY

The data that support the findings of this study are available on request from the corresponding author (S.N.).

Received: 13 December 2019; Accepted: 17 June 2020; Published online: 17 July 2020

REFERENCES

1. Folb, P. I. et al. A global perspective on vaccine safety and public health: the global advisory committee on vaccine safety. Am. J. Public Health 94, 1926–1931 (2004).
2. Barone, P. W. et al. Viral contamination in biologic manufacture and implications for emerging therapies. Nat. Biotechnol. 38, 563–572 (2020).
3. Petricciani, J., Sheets, R., Griffiths, E. & Knezevic, I. Adventitious agents in viral vaccines: lessons learned from 4 case studies. Biologics 42, 223–236 (2014).
4. World Health Organization. Scientific Principles For Regulatory Risk Evaluation On Finding An Adventitious Agent In A Marketed Vaccine (Annex 2). WHO Expert Committee on Biological Standardization. https://www.who.int/biologicals/PDF/ExpertCommitteeSession/S57_98/5792 edición.pdf (2015). (Accessed May 2019).
5. EMEA, E. M. A. ICH Topic Q5A: Quality of Biotechnological Products: Viral Safety Evaluation of Biotechnology Products Derived from Cell Lines of Human or Animal Origin. ICH Harmonised Tripartite Guideline (2006).
6. U.S. Department of Health and Human Services, F. a. D. A. E. Center for Biologics Evaluation and Research. Guidance for Industry. Characterization and Qualification of Cell Substrates and Other Biological Materials Used in the Production of Viral Vaccines for Infectious Disease Indications (February 2010). http://www.fda.gov/BiologicsBloodVaccines/GuidanceComplianceRegulatoryInformation/Guidances/default.htm (2010).
7. Mallet, L. & Gissoni-Lex, L. Need for new technologies for detection of adventitious agents in vaccines and other biological products. PDA J. Pharm. Sci. Technol. 68, 356–362 (2014).
8. Sheets, R. L. & Duncan, P. A. In Vaccine Analysis: Strategies, Principles, and Control (eds Brian K. Nunnally, Vincent E., Turula & Robert D. Sitrin) 389–443 (Springer Berlin Heidelberg, 2015).
9. Gombold, J. et al. Systematic evaluation of in vitro and in vivo adventitious virus assays for the detection of viral contamination of cell banks and biological products. Vaccine 32, 2916–2926 (2014).
10. World Health Organization. Expert Committee on Biological Standardization. https://www.who.int/biologicals/expert_committee/TRS_978_61st_report.pdf?ua=1 (Accessed May 2019).
11. European Pharmacopoeia General Chapter 5.2.14: “Substitution of In Vivo Method(s) by In Vitro Method(s) for the Quality Control of Vaccines”. version 9.3 published in July 2017, creation.
12. European Pharmacopoeia General Chapter 5.2.3: “Cell Substrates for the Production of Vaccines for Human Use”, version 9.0 and updated version 9.3 in July 2017, revision.

13. European Pharmacopoeia General Chapter 2.6.16: “Tests for Extraneous Agents in Viral Vaccines for Human Use”, version 9.3 published in July 2017, revision. Corrected version in Supplement 9.4.

14. Khan, A. S. et al. Report of the international conference on next generation sequencing for adventitious virus detection in biologicals. Biotechnol. J. 55, 1–16 (2018).

15. Khan, A. S. et al. Advanced Virus Detection Technologies Interest Group (AVDTIG): efforts on High Throughput Sequencing (HTS) for virus detection. PDA J. Pharm. Sci. Technol. 70, 591–595 (2016).

16. Lambert, C. et al. Considerations for optimization of high-throughput sequencing bioinformatics pipelines for virus detection. Viruses 10, https://doi.org/10.3390/v10100528 (2018).

17. Ng, S. H. et al. Current perspectives on high-throughput sequencing (hts) for adventitious virus detection: upstream sample processing and library preparation. Viruses 10, https://doi.org/10.3390/v10100566 (2018).

18. Victoria, J. G. et al. Viral nucleic acids in live-attenuated vaccines: detection of minority variants and an adventitious virus. J. Virol. 84, 6033–6040 (2010).

19. Ng, S. H. et al. Preliminary evaluation of next-generation sequencing performance relative to qPCR and in vitro cell culture tests for human cytomegalovirus. PDA J. Pharm. Sci. Technol. 68, 563–571 (2014).

20. Cabannes, E., Hebert, C. & Eliot, M. Whole genome: next-generation sequencing as a virus safety test for biotechnological products. PDA J. Pharm. Sci. Technol. 68, 631–638 (2014).

21. Petty, T. J. et al. Comprehensive human virus screening using high-throughput sequencing with a user-friendly representation of bioinformatics analysis: a pilot study. J. Clin. Microbiol. 52, 3351–3361 (2014).

22. Khan, A. S. et al. A multicenter study to evaluate the performance of high-throughput sequencing for virus detection. mSphere 2, https://doi.org/10.1128/mSphere.00307-17 (2017).

23. Paskey, A. C. et al. Enrichment post-library preparation enhances the sensitivity of high-throughput sequencing-based detection and characterization of viruses from complex samples. BMC Genomics 20, 155 (2019).

24. Mee, E. T., Preston, M. D., Minor, P. D. & Schepelmann, S. Development of a candidate reference material for adventitious virus detection in vaccine and biologicals manufacturing by deep sequencing. Vaccine 34, 2035–2043 (2016).

25. The European Parliament and the Council Of The European Union. Directive 2010/63/EU of the European Parliament and of the Council of 22 September 2010 on the protection of animals used for scientific purposes. Official Journal of the European Union, L 276/233–239 (2010).

26. Charlebois, R. L., Ng, S. H., Gisonni-Leu, L. & Mallet, L. Cataloguing the taxonomic origins of sequences from a heterogeneous sample using phylogenomics: applications in adventitious agent detection. PDA J. Pharm. Sci. Technol. 68, 602–618 (2014).

27. Machnik, G. et al. Sequence analysis of proviral DNA of porcine endogenous retroviruses. Transplant. Proc. 37, 4610–4614 (2005).

28. Tellier, R., Bukh, J., Emerson, S. U., Miller, R. H. & Purcell, R. H. Long PCR and its application to hepatitis viruses: amplification of hepatitis A, hepatitis B, and hepatitis C virus genomes. J. Clin. Microbiol. 34, 3085–3091 (1996).

29. Brussel, A. et al. Use of a new RNA next generation sequencing approach for the specific detection of virus infection in cells. Biotechnol. J. 9, 29–36 (2019).

30. Cheval, J. et al. Adventitious virus detection in cells by high-throughput sequencing of newly synthesized RNAs: unambiguous differentiation of cell infection from carryover of viral nucleic acids. mSphere 4, https://doi.org/10.1128/mSphere.00298-19 (2019).

31. Ma, H., Galvin, T. A., Glasner, D. R., Shaheduzzaman, S. & Khan, A. S. Identification of a novel rhadovirus in Spodoptera frugiperda cell lines. J. Virol. 88, 6576–6585 (2014).

32. Sathiamoorthy, S., Malott, R. J., Gisonni-Lex, L. & Ng, S. H. Selection and evaluation of an efficient method for the recovery of viral nucleic acid extraction from complex biologicals. NPJ Vaccines 3, 31 (2018).

ACKNOWLEDGEMENTS

The authors would like to thank Dr. Rebecca Sheets from the NIH, the QBI and the Vaccine Research Program, Division of AIDS, NIAID who provided the viral strains through Quality Biological, Inc., the Vaccine Research Program (Contract No. HHSN22201100023C) and Jim Gombold for in-depth discussion regarding LOD expression. Editorial assistance with the preparation of the manuscript was provided by Ella Palmer, inScience Communications, Springer Healthcare Ltd., and was funded by Sanofi Pasteur. The authors also thank Jacek Remani and Lauren Rodrigues for support and oversight of the maintenance of the Illumina sequencer, and Sandrine Buisson, for editorial assistance and manuscript coordination on behalf of Sanofi Pasteur. This study was funded by Sanofi Pasteur.

AUTHOR CONTRIBUTIONS

All authors participated in the conception or design of the study; C.L., S.N., and L.G.L. were the scientific leads for obtaining the NIH viral stocks and L.G.L. was head of the Microbiology/Virology platform in Toronto where all of the work was undertaken. R.C., S.S., and S.N. were involved in data acquisition or analysis; all authors contributed to the drafting and critical revision of the manuscript and approved the final version, and are accountable for all aspects of the work presented.

COMPETING INTERESTS

R.C., C.L., L.G.L., and S.N. are employees of Sanofi Pasteur. L.M. and S.S. were employees of Sanofi Pasteur at the time of the study; L.M. is now employed by EDQM–Council of Europe, S.S. is now employed by Turnstone Biologics. R.C., S.N., L.G.L., L.M., and S.S. hold shares in Sanofi.

ADDITIONAL INFORMATION

Supplementary information is available for this paper at https://doi.org/10.1038/s41555-020-0207-4.

Correspondence and requests for materials should be addressed to S.H.S.N.

Reprints and permission information is available at http://www.nature.com/reprints

Publisher’s note Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

© The Author(s) 2020

Open Access This article is licensed under a Creative Commons Attribution 4.0 International License, which permits use, sharing, adaptation, distribution and reproduction in any medium or format, as long as you give appropriate credit to the original author(s) and the source, provide a link to the Creative Commons license, and indicate if changes were made. The images or other third party material in this article are included in the article’s Creative Commons license, unless indicated otherwise in a credit line to the material. If material is not included in the article’s Creative Commons license and your intended use is not permitted by statutory regulation or exceeds the permitted use, you will need to obtain permission directly from the copyright holder. To view a copy of this license, visit http://creativecommons.org/licenses/by/4.0/.