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IABS/DCVMN webinar on next generation sequencing

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ABSTRACT

Next Generation Sequencing (NGS) is a new technology that could overcome some of the limitations of the current viral testing methods for demonstrating the absence of adventitious agents in biologics. This report is for the webinar that was organized by the International Alliance for Biological Standardization (IABS) and the Developing Countries Vaccine Manufacturers Network (DCVMN), held on July 20, 2022, as an introduction to the technical and bioinformatics concepts of NGS and to some of the strengths and limitations of using the technology for those working in vaccine production or development. The current state of scientific knowledge and readiness of NGS to replace or supplement the current viral tests was further discussed in the 3rd Conference on NGS for Adventitious Virus Detection in Biologics for Humans and Animals that was held in Rockville, Maryland, USA, on September 27–28, 2022.

The application of NGS to supplement or replace current *in vivo* and *in vitro* assays in adventitious virus testing during vaccine production is promising; however, assay performance (sensitivity, specificity, and reproducibility) needs to be demonstrated, which may include laboratory and bioinformatics work. Efforts from regulatory authorities, industry, and researchers are ongoing to facilitate validation and establishment of NGS as a new method for virus detection.

Main text

Introduction

The International Alliance for Biological Standardization (IABS, https://www.iabs.org) and the Developing Countries Vaccine Manufacturers Network (DCVMN, http://www.dcvmn.org) organized a joint webinar on the use of next generation sequencing (NGS) technologies for detection of viral adventitious agents in biologicals. NGS (also known as high-throughput sequencing – HTS) is a new technique that allows the sequencing of all nucleic acids (RNA and DNA) in a sample, without prior knowledge of the target sequence and in a short time. NGS is recognized as a powerful technology that could replace current, time-consuming and costly viral testing for adventitious agents. Although this new technology has been used for several years for different applications, it is still novel and with limited, albeit increasing, experience in the field of vaccine production. This webinar was organized to introduce the technical and bioinformatics aspects and challenges of NGS applications for the detection of adventitious viruses in viral vaccines and other biologicals. The challenges and potential applications of NGS in biologics were further discussed in a conference held on September 27–28, 2022 in Rockville, Maryland, USA.

1.2. Introduction to NGS

Sebastiaan Theuns, CEO and co-founder of PathoSense, provided an introduction to NGS technologies. All organisms, plants, animals, and microorganisms, including viruses, have a genetic code, the genome,

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which contains the information for the production and function of proteins. For most organisms, the genetic code is DNA, but in some viruses, it is RNA. The genetic code can be revealed by nucleotide sequencing. This can be done by Sanger sequencing, which can give reads of 500–1000 base-pairs (bp). However, nowadays, with the use of NGS, millions of base-pairs can be obtained in one analysis. Many developers are providing NGS technologies, leading to strong competition based on the technology, accuracy, output (duration of runs), and read length. Regarding the latter, two strategies are available: the short-read technology, provided by Illumina, IonTorrent, MGI, Genapsys, Element Biosciences, and Ultima Genomics; and the long-read technology, provided by Oxford Nanopore Technologies and PacBio. Some short-read technologies involve: bridge amplification followed by sequencing by synthesis using fluorescently labeled nucleotides (Illumina), rolling circle amplification and sequencing of DNA nanoballs (MGI), and ion semiconduction (IonTorrent). Long-read platforms use techniques such as single-molecule real-time sequencing based on fluorophores (PacBio), and nanopore sequencing technology (Oxford Nanopore Technologies). Long-read platforms allow for a more accurate assembly of the genome ("by piecing the long reads together") than using short-read platforms. Many providers supply instruments with varying throughput for different applications, from benchtop to mega systems [1].

The main problem with traditional molecular-based pathogen detection/adventitious agent testing is that it generally targets pathogens. This *a priori* selection will only detect known organisms and will not provide a full overview of potential agents that may be present in a sample. Moreover, some pathogens, especially RNA viruses, can rapidly change in their sequences. In addition, the genomes of pathogens are very small compared to the human genome (1.5–150 kb for viruses, 1 Mb for *Mycoplasma*, 5 Mb for *Escherichia coli*, compared to 3.2 Gb for the human genome). Hence, pathogen enrichment is essential for appropriate detection but requires detailed and labor-intensive procedures.

NGS has a variety of applications. The capacities of NGS for viral diagnostics of enteric diseases in swine were investigated [2]. Diarrheic feces of a one-week-old piglet were analyzed and *Bacteroides, Escherichia,* and *Enterococcus* phages were found. Incidentally, porcine kobuvirus was discovered in the feces for the first time in Belgium. Furthermore, NGS allowed investigation of the molecular epidemiology of porcine parvovirus type 1 (PPV1), which results in stillbirth, mummification, embryonic death, and infertility in pigs [3]. PPV1 has evolved at a rate of 4.7×10^{-5} nucleotide substitutions per site per year. Extensive sequencing by NGS allowed evaluating and reassessing the current PPV1 VP1-based classifications, providing evidence for the existence of four relevant phylogenetic groups. Finally, NGS was used to implement a first genome-wide association study approach to identify genetic markers linked to antimicrobial resistance in *Mycoplasma bovis*, resulting in identification of potential genetic markers for macrolides and enrofloxacin [4].

In conclusion, NGS can help obtain faster and better results without the need for prior selection, leading to better advice on product safety. Because NGS is an innovative field and still evolving, advances in technology may lead to challenges for regulatory bodies. Beyond adventitious agent testing, NGS may also be of value in the selection of lead biological molecules in antibody drug discovery and in pharmacovigilance thanks to the rapid detection of mutations, recombination, and reassortment.

1.3. Reference materials for NGS for adventitious virus detection

Arifa Khan, Supervisory Microbiologist in the Division of Viral Products at the Office of Vaccines Research and Review (OVRR) in the U. S. Food and Drug Administration (FDA), provided an overview of the current assays recommended for adventitious virus detection and the challenges and advantages of using NGS.

Currently, adventitious viruses are mitigated using an integrated strategy, that encompasses prevention, clearance, and testing. This includes identifying potential sources of adventitious virus introduction, evaluating risks of contamination, using qualified materials and, depending on the product, incorporating robust viral clearance steps during manufacturing. Extensive tests for known and unknown agents are carried out in the starting materials and at different stages of the manufacturing process, using various sensitive and broad detection assays. The currently recommended assays for viral detection, including general and species-specific tests, have generally been effective in demonstrating the absence of adventitious viruses for product safety. However, these methods have limitations: *in vitro* and *in vivo* assays may take long to get a result (cell culture >28 days; animal-based >18 days), and molecular assays are designed using available known virus sequences. Additional testing strategies (such as chemical induction) have been developed for broad virus detection in novel cell substrates [5], but these are integrated with conventional methods that detect known viruses.

NGS technologies can generate sequences of all nucleic acids present in a sample and therefore broadly detect known and unknown viruses without prior sequence knowledge. Potential applications of NGS for adventitious virus detection in biologics have been demonstrated by the finding of porcine circovirus type 1 (PCV1) in a licensed rotavirus vaccine [6] and the discovery of a novel rhabdovirus in the Sf9 insect cell line used for baculovirus-expressed products [7]. In both cases, extensive testing had previously been done using the currently recommended assays to demonstrate the absence of adventitious viruses.

The main challenges of NGS applications currently reside in the standardization and validation of assays. Appropriate reference viruses and standards are needed to demonstrate the efficiency of the different steps and the sensitivity and specificity of the method. On the bioinformatics side, data analysis pipelines need optimization. Reference datasets and parameters for assembly need to be established, as well as the criteria for acceptable quality of reads. The database used in the bioinformatics analysis needs to contain diverse viral sequences for novel virus detection and be correctly annotated for accurate results. The format for secure data submission, storage, and transfer needs to be defined. Finally, a follow-up strategy needs to be established to confirm "true" hits and determine the biological relevance and significance of a positive signal.

The FDA and the industry have ongoing efforts to address these challenges. One is the Advanced Virus Detection Technologies Interest Group (AVDTIG), aimed to advance the tools for the next generation of viral risk evaluation by providing an informal, scientific forum for discussions and scientific collaborations [8]. The group includes international participants from industry (vaccine, therapeutics, and gene therapy), regulatory and other government agencies and national authorities, academia, service providers and technology developers, who meet by teleconference every other month. Different focus groups target priority areas such as: sample selection/preparation/processing, viral reference reagents, databases with current focus on the development of a complete and correctly annotated public reference virus database, optimization of bioinformatic pipelines for NGS analysis, and follow-up strategies to confirm a hit. Among the efforts toward NGS standardization and reference materials, Dr. Khan's laboratory collaborated in the first spiking study to evaluate breadth and sensitivity of virus detection by different NGS platforms [9]. The study demonstrated that similar results for virus detection could be obtained regardless of using independent protocols for sample extraction and processing, different NGS platforms, and bioinformatics analysis pipelines in the NGS workflow. Furthermore, Dr. Khan's laboratory developed five virus stocks, now adopted as World Health Organization (WHO) International Reference Reagents for Adventitious Virus Detection in Biological Products by High-Throughput Sequencing [10], and has generated a complete Reference Viral DataBase (RVDB) that includes viral, viral-related, and viral-like sequences (https://rvdb.dbi.udel.edu) [11].

NGS can improve viral safety testing by increasing efficiency (reducing time); avoiding ethical challenges (reducing animal use); and

defining the limit of detection, specificity, reproducibility, and accuracy. NGS can play a role in mitigating the risk of adventitious virus introduction in materials and monitoring their absence during production. However, assays must be validated prior to use for testing biological products for human use. This includes assessing accuracy, precision, limits of detection, limits of quantification, specificity, linearity and range, ruggedness and robustness, and system suitability. In the OVRR, submissions for investigational viral vaccines have included use of NGS for complementing, supplementing or replacing one or more of the conventional assays for adventitious virus testing. The urgent need to accelerate SARS-CoV-2 vaccine development due to the COVID-19 pandemic has resulted in an increase in the number of requests from different sponsors to use NGS for cell line characterization and adventitious virus testing to ensure product viral safety. Due to the complexity of the technology, OVRR has offered technical discussions to sponsors and CROs for using NGS for adventitious virus detection in viral vaccines and provided guidance on regulatory expectations for NGS submissions.

1.4. The European Directorate for the quality of Medicines & Healthcare (EDQM) achievements and perspectives on NGS

Laurent Mallet and **Gwenael Cirefice**, of the European Directorate for the Quality of Medicines and HealthCare (EDQM), discussed the evolution of the European Pharmacopoeia (Ph. Eur.) requirements for the extraneous agents testing of vaccines. EDQM is a Council of Europe (CoE) directorate that aims to facilitate access to quality medicines and healthcare and is responsible for the elaboration of the Ph. Eur. The Ph. Eur. lays down common, compulsory quality standards for all medicinal products in Europe and is legally binding in all 39 CoE states who have signed the Convention on the Elaboration of a European Pharmacopoeia [12]. The Ph. Eur. needs to keep pace with the regulatory needs of licensing, control, and inspection of authorities in the public health area, industrial constraints, and technological and scientific advances. It comprises three main types of documents: general monographs, which are mandatory and provide quality requirements to classes of products; individual monographs, which are also mandatory and are based on approved specifications and validated analytical procedures; and general chapters, which provide guidance and recommendations for analytical procedures and are not mandatory but can become mandatory when referred to in a monograph.

The Ph. Eur. has been evolving in terms of extraneous agents testing for vaccines. Among the drivers for change were: (a) the identification of a rotavirus vaccine contamination by porcine circovirus [6]; (b) the emergence of broad molecular methods; (c) the revised WHO TRS 978 Annex 3 to include the risk assessment strategy and new methodologies, including NGS; (d) the convergence with the FDA Guidance for Industry on testing methodologies; (e) the 3Rs context in Europe for replacement, refinement, and reduction of animal use, particularly the European Convention for the Protection of Vertebrate Animals used for Experimental and Other Scientific Purposes (Council of Europe) and EU Directive 2010/63/EU [13], (f) an EDQM survey among vaccine manufacturers and Contract Research Organizations (CROs) regarding contamination cases over a period of 10 years conducted in 2012, and (g) several publications highlighting gaps in compendial tests, particularly the poor sensitivity of *in vivo* tests [14,15]. These led to a major update of the Ph. Eur., in July 2017, where two major chapters were revised, and a new chapter was introduced.

The existing chapters 5.2.3, *Cell substrates for the production of vaccines for human use*, and 2.6.16, *Tests for extraneous agents in viral vaccines for human use*, were revised to introduce the concept of risk assessment. The testing strategy for viral extraneous agents should be based on an evaluation of the risks for a given product/process, and include a package of tests able to detect the relevant families of extraneous agents according to the risk evaluation. The panel of tests is to be adapted depending on the potential contaminants for each product. Both chapters allow the use of broad molecular methods (NGS or HTS, which is the preferred term used in the Ph. Eur.), and remove *in vivo* tests in adult mice and guinea pigs. Both chapters establish that the testing strategy should be based on risk assessment, considering that molecular methods such as NGS/HTS can be introduced, and any maintained *in vivo* tests need to be justified.

The new Chapter 5.2.14, *Substitution of in vivo method(s) by in vitro method(s) for the quality control of vaccines*, was elaborated to facilitate the transition to *in vitro* methods, such as HTS, for veterinary and human vaccines. This chapter provides guidance on how to introduce alternative *in vitro* methods where a head-to-head comparison is not possible and envisages the concept of substitution as an alternative approach for the replacement of *in vivo* methods. It is focused on the scientific rationale behind the *in vitro* methods and validation package.

Chapters 5.2.3 and 2.6.16 foresee the application of HTS methods in the testing strategy for extraneous/adventitious agents; however, no regulatory document provides guidance for the validation of HTS methods currently. The availability of regulatory standards, including validation guidelines, will help accelerate the global adoption of the new technology. Currently, HTS is foreseen to be introduced in the revised ICH Q5A guideline on viral safety evaluation of biotechnology products, and the FDA has recently developed panels of model viruses, which have been adopted by the WHO as reference standards for HTS [10]. Therefore, a new chapter 2.6.41, *High Throughput Sequencing for the detection of extraneous agents in biological products*, is being elaborated as a non-binding general chapter, which will describe HTS methods and establish guidelines for their validation. This chapter is under elaboration by a Ph. Eur. HTS Drafting Group, comprising an international group of regulators, Official Medicines Control Laboratories (OMCLs), and industry experts.

1.5. Employing NGS for public health

Ravneet Bhuller, Genomic Data Scientist at the Medicines & Healthcare products Regulatory Agency (MHRA), discussed MHRA's experiences using NGS technologies and their applications in public health. MHRA's main roles include regulatory and laboratory activities. MHRA is the main regulatory body in the UK that regulates medicines, medical devices, and blood transfusion components. MHRA aims to strengthen patient safety, such as in the recent approval of the Valneva COVID-19 vaccine. Laboratory activities are mainly performed at the South Mimm's site, former National Institute for Biological Standards and Control (NIBSC, now absorbed into MHRA). These include the development of WHO-approved international standards that assure the quality of biological medicines and diagnostics, the independent batch release control testing of biological medicines and vaccines, and underpinning regulatory research (e.g., Polio research in wastewater https://www.gov.uk/government/news/poliovirus-detected-in-sewage-from-north-and-east-london).

NGS technologies have greatly reduced the time and costs spent on sequencing as compared to the first-generation sequencing technologies. However, the applications of second-generation sequencing (short-read NGS) were limited by the difficult assembly of reads, particularly in the case of large or complex genomes with highly repetitive regions. Third-generation sequencing (long-read NGS) platforms can overcome these limitations, and new platforms are expected to evolve and address the remaining challenges.

One of the main challenges with NGS data during analysis is reproducibility, as the same application deployed in different hardware or different environments (Linux, Windows, macOS, or Cloud) produces different results [16]. New programming languages, such as Nextflow, help to reduce the effects of the hardware in the NGS data analysis and improve reproducibility [17]. A second challenge is the exponential increase in the amount of data being produced using NGS technologies, which raises issues for the handling, storage, transfer, and analysis of the data, only partially resolved by Cloud technology. The third main challenge is the availability of numerous NGS assays and bioinformatic

tools that produce different results. Therefore, best practices are required to harmonize results. Reference reagents are useful to resolve this issue, as they can help calibrate diagnostic assays and optimize bioinformatic pipelines.

NGS technologies have been applied at MHRA for different purposes. A study with the participation of 16 different laboratories revealed that the wide range of methods in use can produce significantly different results, highlighting the importance of reference reagents for result comparability and harmonization [18]. As part of the national surveillance strategy, NGS was applied to detect and characterize SARS-CoV-2 variants from February 2020 to March 2022 and to develop a reference panel for SARS-CoV-2 variants of concern. NGS has allowed to detect human enteroviruses in sewage [19,20]. MinION Nanopore sequencing allowed to directly detect and sequence polioviruses, speeding up the process, providing capacity to smaller labs and field teams, and characterizing diversity in single samples by deep sequencing [21]. In cancer genomics, different laboratories obtained different results when using different platforms and analysis pipelines to identify single nucleotide variants (SNV) in five candidate genes. Reference reagents were prepared to calibrate the analyses and improve diagnostics [22]. Scientists from the MHRA have further developed standards to harmonize NGS microbiome characterization methodologies and improve result accuracy and quality control of cell therapy products [23].

In summary, NGS is a powerful, agnostic-based, technology for a variety of applications including adventitious agent testing and diagnosis of infectious diseases, cancer, and other chronic diseases. However, reproducibility needs to be assured both in the wet laboratory and in bioinformatic pipelines for implementation of NGS in biologics. The use of reference reagents contributes to data harmonization and the generation of more reliable results.

2. Discussion

2.1. How can NGS detect live adventitious agents in contrast to conventional methods?

Arifa Khan responded that NGS detects all viruses in a sample, including the vaccine/biological product. It is a nucleic acid-based test so it cannot discriminate between live or dead agents. However, a contaminant may be further analyzed at the bioinformatic analysis stage by a variety of approaches to evaluate presence of a full-length genome that could potentially indicate presence of an infectious virus. Adventitious virus detection can be enhanced by "subtracting" the vaccine virus or any other expected sequence including host cell nucleic acid from the total sequences obtained.

2.2. How do you address the high false-positive and cross-contamination rate in highly sensitive platforms like the illumina platform, particularly when facing regulatory authorities?

Laurent Mallet and **Gwenael Cirefice** clarified that the Ph. Eur. already describes the need to have a follow-up investigation after HTS detection, and further guidance on this is planned to be included in the new HTS chapter. **Arifa Khan** agreed and added that the strength of NGS is to facilitate detection, and this is just the first step. After a sequence is identified, further investigation on the biological significance of the sequence needs to be carried out to understand if the sequence is associated with a viral particle and whether it is infectious. Signal detection does not imply closing the facility, but it needs to be investigated, and reagents need to be tested using the knowledge acquired from the NGS analysis. The analysis includes follow-up testing with the collaboration of virologists and bioinformaticians. It is a complex but powerful technology. The AVDTIG is preparing a publication that will detail follow-up strategies for NGS signals, including lab-based procedures and bioinformatics.

2.3. Could nanopore sequencing replace other NGS platforms?

Sebastian Theuns clarified that Nanopore sequencing can be as accurate as other platforms if sufficient reads are used. The opinion that Nanopore is not an accurate technology should change, as Nanopore platforms are reaching the same accuracy values as competitors.

2.4. How do you differentiate the adventitious infectious from non-infectious agent sequences? How do you differentiate between endogenous retroviruses from active viruses?

Arifa Khan explained that whatever nucleic acids are in the sample will be sequenced and detected. Pre-treatments to remove non-encapsidated RNA or DNA have not been extensively investigated yet, and preliminary data show that RNase and DNase treatments may impact the integrity of some viruses; thus, these strategies may not be ideal for adventitious virus detection. Another method under investigation is the ultracentrifugation of the particles to discard non-particle-associated nucleic acids. However, none of these methods allow to distinguish infectious from non-infectious particles. The strength of the method is in the initial detection, and then the follow-up investigation determines whether the signal detected is infectious. Mapping the reads against a reference genome can help to evaluate if the data obtained are full-length. However, this is also not completely reliable because the number of reads required for a positive result has not been determined. The only reliable strategy is to conduct a thorough follow-up investigation.

To detect endogenous retrovirus sequences, strategies may be to include the database with all the sequences available for endogenous retroviruses in the analysis. Then, during follow-up, the level of detection needs to be monitored throughout the production. The final proof would be to do an infectivity assay.

2.5. Can NGS replace the methods prescribed in the WHO/TRS CFR pharmacopoeia?

Laurent Mallet believes it is possible to propose a replacement of methods, particularly *in vivo* methods, as already described for the Ph. Eur. However, full validation is required to demonstrate the sensitivity and breadth of detection of the method. This is foreseen in the Ph. Eur. revised chapters, and the new chapter will describe in more detail the validation approach of these assays. Already since 2010, NGS methods have been introduced in the WHO TRS (978 Annex 3), and all other WHO recommendation documents in the field of vaccines revised since then have addressed them. **Arifa Khan** added that the urgency for rapid vaccine development during the pandemic pushed NGS to the forefront in regulatory submissions. Different considerations were raised for different NGS applications, and the use of reference viruses was valuable.

2.6. Why not include bacterial agents in validations and rely on qPCR?

Laurent Mallet responded that for the Ph. Eur, it was decided to restrict the applications and chapters to viral agents, as that was the major gap in the analytic breadth of detection. For bacterial adventitious agent detection, there is a need to ensure that all the reagents included in the NGS pipeline do not introduce bacterial DNA, and it is difficult to remove all other bacterial DNA from these reagents.

2.7. Depending on the cell substrate, would it be possible to develop a list of agents of concern to screen for?

Arifa Khan replied that it is difficult to have a list of agents of concern because this is constantly evolving. The risk assessment is determined by scientific and regulatory knowledge and industry experience to define the agents of concern, considering, for example, the host

from which the cell line was derived, the reagents, or the susceptibility of a cell substrate to virus infection. That is why NGS is valuable.

2.8. What about the pipelines for analyses of NGS which rely on good manufacturing practice (GMP) applications?

Laurent Mallet answered that what has been described in the European Pharmacopoeia is for GMP applications, e.g., testing for vaccines, cell substrates, cell banks, viral seeds, and viral harvest. NGS is a complex technology that involves different stages and challenges that do not exist in other methods used in the industry. However, the change control must be followed, for example, when updating reference databases. **Arifa Khan** added that the approach has been to validate each stage of the pipeline instead of the entire workflow. Thus, when something changes in one stage, only that stage will need revalidation.

3. Conclusions

This webinar provided an overview of the technical concepts of NGS for adventitious agent testing in biologicals. The main advantages and challenges of using NGS have been presented, as well as a summary of the status of relevant regulatory documents. NGS is a promising technology for vaccine manufacturers, either to supplement or to replace the currently used methods for the detection, characterization, or identification of adventitious viruses. However, validation guidelines and description of the technology are needed to further support its implementation and standardization. Updates on the use of NGS for adventitious virus detection in biologicals were further discussed in the Conference co-organized by IABS, EDQM, and the FDA held in Rockville, Maryland, USA, on September 27–28, 2022 (meeting report is in preparation).

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