Regulatory Expectations for New Analytics: *Considerations for NGS Standardization for Adventitious Virus Detection*

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Disclaimer

This presentation is an informal communication and represents my own best judgment. The material in this presentation and my comments do not represent FDA policy regarding use of NGS for virus detection.

Outline of Talk

- **Potential for NGS to replace/supplement adventitious virus detection assays**
- **Introducing new assays for qualification of biological materials for production**
- **Challenges of NGS for standardization and current efforts**

Adventitious viruses are a major safety concern in biologics, especially in live products, such as viral vaccines and gene therapies, where manufacturing generally cannot include viral inactivation or removal steps.

Currently recommended assays:

- **General detection assays**
  - ***In vitro* cell culture tests in cell lines of 3 species (same as cell substrate, monkey, human)**
  - ***In vivo* assays (adult mice, suckling mice, embryonated hens' eggs)**
  - **Transmission electron microscopy (TEM)**
  - **Reverse transcriptase assay for retroviruses (PERT)**
- **Species-specific Assays**
  - **Tests for animal viruses e.g. bovine, porcine (9CFR 113.47 and 113.53)**
  - **Antibody-production assays for rodent viruses (MAP *including LCMV challenge*, HAP, RAP)**
  - **Assays for known viruses (PCR, DNA hybridization, Infectivity, antibody detection)**
- **Additional Assays for Novel Cell Substrates** *(Recommended case-by-case)*
  - **Extended PCR assays**
  - **Oncogenicity assays:** Tumor-inducing viruses
  - **Chemical induction assays:** Endogenous retroviruses, latent DNA viruses

Currently recommended assays can "miss" detection of novel and even some known viruses

➢ ***Need for advanced, broad, adventitious virus detection methods***

- ❑ **NGS has the ability to detect known and unknown viruses**, without prior sequence knowledge.
- ❖ **Potential applications of NGS in biologics has been demonstrated**
  - Finding porcine circovirus type 1 (PCV1) in a licensed rotavirus vaccine *(Victoria et al., 2010)*
  - Discovery of a novel rhabdovirus in the Sf9 insect cell line used for baculovirus-expressed products *(Ma et al., 2014).*
  - ***In both cases, extensive testing was done using currently recommended assays to demonstrate absence of adventitious viruses.***

NGS for Broad Adventitious Virus Detection

- **Known, unknown, and unexpected viruses**
  - **Tumor-inducing viruses** (*in case of tumor cell lines*)
  - **Latent viruses** (*infectious but silent and can become active*)
    - **DNA and RNA viruses**
    - **Endogenous retroviruses**
  - **Occult viruses (***known, unexpected***)**
  - **Novel viruses (***unknown***)**

- **Reverse transcriptase (RT) activity**
  - **Produced constitutively from avian and insect cells** (endogenous retroviruses: retroviral particles/retrotransposons)

Potential of NGS for Virus Detection in Biologics

- **Replacement assay (*in vivo* and PCR assays)**
  - ***In vivo* AV assays** – NGS can provide defined sensitivity and breadth of virus detection
  - **PCR assays** – NGS can have similar or greater sensitivity than PCR assays; broader virus detection; single assay
- **Supplementary assay (*in vitro* assays)**
  - **Cell substrate characterization** – particularly in case where there are concerns for occult and novel viruses
  - ***In vitro* AV assays** – particularly in case of assay interference due to lack of effective neutralization of vaccine virus; as a read-out to reduce assay time
  - **Follow-up of a positive result *(as for any nucleic acid-based detection assay)* – NGS data may help design a "custom" assay to determine if signal due to infectious virus**

Introduction of Improved Assays

- Increased efficiency (time)
- Ethical (reduce animal use)
- Superiority (LOD, specificity, repeatability, accuracy)

Example of Improved Analytics for Adventitious Agents

- **Mycoplasma testing**
  - Conventional testing
    - Agar/broth and indicator cell culture methods
    - 4-6 weeks
    - Labor intensive
    - Some strains not detected or with difficulty
  - Replacement assays
    - For *Mycoplasma* testing of cells and virus seed testing……NAT may be used as an alternative after suitable validation (*E.P. 9.0 section 2.6.7, 2017*)
    - …for live virus vaccines and inactivated virus vaccines ……proposed rule would remove the specified test for the presence of *Mycoplasma* to provide flexibility for accommodating new and evolving technology and capabilities……..These newer technologies can result in higher sensitivity and specificity of *Mycoplasma* detection and could reduce the time required to complete testing for *Mycoplasma*. (*Proposed Rule by FDA April 2, 2019 Federal Register*)

Introduction of New Assays for Adventitious Virus Testing*

Recommended testing has been updated over time

- **Availability of new methods for more sensitive virus detection**
  - PERT assay for retrovirus particles *(validated assays available; recommended by OVRR to replace traditional RT assay)*
- **Novel virus discoveries**
  - PCR assays for virus-specific detection *(validated assays available or developed in-house; recommended or sponsor-initiated)*
- **Use of novel cell substrates** *(validated assays not available – recommended on a case-by-case basis by OVRR)*
  - Oncogenicity assays for detection of oncogenic viruses *(positive controls not available)*
  - Chemical induction assays for latent or endogenous virus detection *(positive controls available but not species-specific)*

*** CBER Cell Substrate Guidance for Viral Vaccines**

U.S. Department of Health and Human Services Food and Drug Administration
Center for Biologics Evaluation and Research [February 2010]

Guidance for Industry

Characterization and Qualification of Cell Substrates and Other Biological Materials Used in the Production of Viral Vaccines for Infectious Disease Indications

This guidance represents the Food and Drug Administration's (FDA's) current thinking on this topic. It does not create or confer any rights for or on any person and does not operate to bind FDA or the public. You can use an alternative approach if the approach satisfies the requirements of the applicable statutes and regulations. If you want to discuss an alternative approach, contact the appropriate FDA staff. If you cannot identify the appropriate FDA staff, call the appropriate number listed on the title page of this guidance.

- Provides guidance for the characterization and qualification of **cell substrates, viral seeds, and other biological materials (including vaccine intermediates)** used in manufacture of viral vaccines for human use.

- **WHO TRS 978 Annex 3 (2010, *pub 2013*)** "It is probable that application of methods of this type (*nucleic acid based methods with broad detection capabilities*) will be expected or required by regulatory agencies in future. At present the methods have not been evaluated for sensitivity and specificity and should be thought of as powerful investigational tools that can reveal issues that can be explored by more established methods"

- **Ph. Eur. 9.4, Chapter 2.6.16. (2017) Tests for Extraneous Agents in Viral Vaccines for Human Use.** "New, sensitive molecular methods with broad detection capabilities are available. These include HTS,…. These methods may be used either as an alternative to in vivo tests and specific NAT or as a supplement/alternative to in vitro culture tests based on risk assessment and with the agreement of the competent authority.
- **Ph. Eur. 9.3. Chapter 5.2.3. (2017**) "Cell Substrates for the production of vaccines for human use"
- **Ph. Eur 9.3. Chapter 5.2.14 (2017)** "Substitution of *in vivo* method(s) by *in vitro* method(s) for the quality control of vaccines

….an appropriate panel of representative, well-characterized model viruses should be used to assess the ability of the new method to detect viruses……

animals. The implementation of such new molecular methods as substitutes for *in vivo* methods requires a comparison of the specificity (breadth of detection) and the sensitivity of the new and existing methods. For this purpose, an appropriate panel of representative, well-characterised model viruses should be used to assess the ability of the new method to detect viruses that are (or are not) detected by the *in vivo* methods, and to determine if the sensitivity is at least equivalent to the sensitivity of the *in vivo* methods. This last element is particularly complex since these new molecular methods do not detect the same characteristic of the viral contaminant (genome for molecular methods versus infectious virus for *in vivo* methods) and also since no or limited validation data exist for the *in vivo* methods. It should also be emphasised that the outcome of the new molecular methods is not the final result since the detection of a genome or fragments of a genome does not necessarily indicate the presence of an infectious virus.

Introducing New Assays for Regulatory Applications

In general, molecular assays like virus-specific PCR assays are easier to validate compared to biological assays (fewer variables)

- **It is recognized that NGS is more challenging due to the high complexity of the technology**

General NGS Workflow for Adventitious Virus Detection

(PRE-TREATMENT)	• Reduction of “free” nucleic acid using nuclease • Enrichment of particles by WGA/filtration/size selection/ultracentrifugation
NUCLEIC ACID EXTRACTION	• Cells • Cell lysate • Supernatant (cell-free)
LIBRARY PREPARATION	• rRNA depletion/polyA+ selection • cDNA synthesis • Target-specific amplification • Fragmentation
SEQUENCING	• Short-read and Long-read platforms
BIOINFORMATICS	• Assembly programs • Analysis tools • Databases

Challenges for Using NGS for Virus Detection in Biologics *(Identified in various public meetings: since 2009)*

❑Standardization and Validation

- **Appropriate model viruses (*for spiking studies*)**
  - **Efficiency of the different steps involved in the methodology**
  - **Sensitivity and specificity**

❑Bioinformatics

- **Data analysis**
  - **Pipeline optimization**
    - Criteria for acceptable quality of reads
    - Parameters for short read assembly; hybrid assembly to correct high error-rate currently seen in long-read sequencing
    - Strategies to identify a novel virus that has minimal nucleic acid sequence homology to known viruses
  - **Development of a complete and correctly annotated, publicly available, Reference Virus Database**
- **Data submission, storage, and transfer**
  - **Format**
  - **Security**

❑Follow-up strategy

- **Confirmation of a "true" hit**
- **Determination of biological relevance and significance of a positive signal**

FDA/Industry Efforts on NGS for Virus Detection in Biologics

Advanced Virus Detection Technologies Interest Group (AVDTIG)

(PDA-sponsored "Users Group" in Oct. 2012; "Interest Group" since 2014)

"Mission" – To advance next generation tools for viral risk evaluation by providing an informal, scientific forum for open discussions and scientific collaborations

- **Co-chairs**
  - **Arifa S. Khan**: FDA, U.S.
  - **Dominick Vacante**: Janssen R & D, U.S.
  - **Jean-Pol Cassart**: GSK, Belgium
  - **Keisuke Yusa**: National Institute of Health Science, Japan
- **Open public participation: > 150 scientists (U.S., Europe, Japan) from industry (vaccine and therapeutics, gene therapies), regulatory and other government agencies and national authorities, academia, CROs, and others**
  - Meetings/discussions by t-con every other month
  - Focus subgroups with additional meetings

PDA J Pharm Sci and Tech 2016, 70 591-595

AVDTIG Subgroups

2018

AB

- Subgroup A: Sample selection/preparation/processing (***evaluating the different approaches to sample preparation for detection viral adventitious agents by HTS***) Siemon.Ng@sanofipasteur.com
- Subgroup B: Virus standards and reference materials (***identify types of virus reference materials for spiking studies based upon different potential applications***) Jean-Pol.Cassart@gsk.com

C

- Subgroup C: Databases evaluations and development of a complete and correctly annotated, publicly available virus reference database (***identify criteria for developing and testing a representative, complete reference viral database for detection of known and novel viruses***) Arifa.Khan@fda.hhs.gov (and Stephane.Cruveiller@pathoquest.com – *since 2018*)

DE

- Subgroup D: Bioinformatics pipelines analysis (***understanding what makes a good pipeline and how it should be tested***) Christophe.G.Lambert@gsk.com
- Subgroup E **(formed in 2016):** Follow-up strategies to confirm the identity of a "hit" (***to explore best practices for investigations and risk assessment of an HTS signal***) Robert.Charlebois@sanofipasteur.com

PDA J Pharm Sci and Tech 2016, 70 591-595

KHAN Lab Efforts Towards NGS Standardization

- Collaborative study to evaluate virus detection by different NGS platforms *(initiated early-2014)*
  - The first (pilot) virus spiking study with 4-5 model viruses completed (FDA, GSK, and Sanofi)
  - Published (*mSphere, Sept/Oct 2017*) and datasets available in NCBI

- Development of virus reference stocks *(initiated early-2015)*
  - Five large scale, well-characterized virus stocks have been prepared at ATCC to evaluate performance of NGS platforms for standardization

- Generation of a complete Reference Virus Database (RVDB) *(initiated in mid-2013)*
  - In-house lab efforts in consultation with AVDTIG scientists have resulted in development of a word-based viral database that includes all viral, viral-related, and viral-like sequences
  - Publicly available – current release v16.0 (May 29, 2019) –at GWU HIVE https://hive.biochemistry.gwu.edu/rvdb
  - *Moving to University of Delaware in Oct 2019!*

AVDTIG Discussions*: NGS Virus Reference Materials for Spiking Studies to Assess Sample Preparation and Processing (2014 ->)

Nucleic acid Extraction

Library Preparation

- **Whole Virus *(Live)***
  - **Purified (Spiking Study I)**
  - **Non-purified**
    - **Mimick samples from production**
- **Infected cells**
  - **Active virus production**
  - **Virus RNA expression**
  - **Latent virus**

Library Preparation

- **Viral Nucleic Acids**
  - **Pre-extracted DNA/RNA**
  - **Synthetic constructs (plasmids, RNA)**

***Jean Pol Cassart: Subgroup B**

Considerations for Assay Validation

You should demonstrate the reliability of assays or tests, in the context of intended use, for demonstrating absence introduction of adventitious agents in your product.

- Assays related to assurance of safety should be scientifically valid (for example, by formal validation and/or inclusion of appropriate controls or standards) prior to initiation of clinical trials. This may include:
  - Development of relevant controls (*positive and negative)*
  - Determination of sensitivity and specificity
  - Demonstration of precision (*reproducibility and repeatability*)
  - Evaluation of assay robustness (*change of assay conditions and reagents*)
  - Demonstration of the reliability of the assays (e.g. interference of sample matrix to the assay's intended use)
  - More details on the validation of analytical assays and statistical analyses are described in ICH Q2(R1)
- Availability of assay
  - Establishment of method in-house or through CROs
    - need publicly available standards

Some factors that can influence sensitivity and breadth of virus detection by NGS

❑ Sample preparation and processing

- **Test volume (sup vs cells)**
- **Extraction efficiency of different virus structure types**
- **cDNA synthesis of different virus genome types / library preparation**
- **Enrichment steps for viral nucleic acid (nuclease treatment, polyA+ selection, ribosomal depletion in case of residual host cell sequences)**
- **Controls (reagents, method)**

❑ Sequencing platform

- **Selection of sequencer to provide sufficient reads to detect a low level virus - need to consider coverage for different genome sizes and sample types (supernatant, transcriptome/genome)**
- **Need to consider error rate of sequencing technology and detection of a novel virus: short reads vs long reads**

❑ Bioinformatics

- Strategies for detection of known and novel viruses (nucleotide vs amino acids, programs/tools, reads vs contigs, criteria and parameters for runs)
- Databases (*should contain viruses from all species that could potentially introduce adventitious virus at any step of the production*)
- Unmapped reads?
- Re-analysis?

Conclusions

- **Currently, NGS can be useful for supplementing the conventional assays for novel virus detection**
- **NGS may be considered as an alternate method for replacing some conventional assays based upon justification for suitability and fit for purpose (*may be considered on a case-by-case basis*).**
- **Efforts for developing, *publicly available,* NGS reference materials (physical standards and electronic datasets and databases) should continue to provide confident and accurate results for broad method implementation**
- **Need for early discussions between industry, regulatory authorities, and technology providers to identify and address scientific challenges since the technology is still maturing**

Workshop Goals

- Identify currently used viral standards
- Identify public availability
- Identify the current gaps in viral standards
- Prioritize the needs
  -> Opportunities for collaborations and resource sharing

Thank You!

REGISTRATION INFORMATION

https://2nd-next-generation-sequencing-ghent-2019.iabs.org/

Scientific Committee

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Sebastiaan Theuns, Ghent University / PROVAXS
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Laurent Mallet, Sanofi Pasteur
Gerald Schumann, Paul-Ehrlich Institut (PEI)
David Mackay, Advisor Veterinary Vaccinology
Joseph Victoria, Boehringer-Ingelheim

- **Bring together industry, academia, technology providers, and international regulatory bodies to discuss current status of NGS for adventitious virus detection in biologics**
- **Present ongoing efforts on standardization and validation of the technical and bioinformatics steps in NGS for its applications in characterization and safety evaluation of biologics, including human and animal vaccines.**
- **Develop a scientific consensus regarding readiness of NGS for detection of adventitious viruses in biologics.**

SPONSORSHIP INFORMATION

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