A Regulatory Perspective on Using Next Generation Sequencing for Adventitious Virus Detection in Biologics

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Overview of Talk

❖ Background for NGS moving into the biologics space
❖ NGS efforts and progress toward applications in biologics
❖ Highlights from the Nov 13-14, 2019 IABS conference on NGS for Adventitious Virus Detection in Biologics (Ghent, Belgium)
❖ OVRR’s current thinking about NGS for adventitious virus detection
❖ NGS considerations for updating ICH Q5A(R2)
Need for Advanced Virus Detection Technologies

- Use of **novel cell substrates** for development of new vaccines against emerging and re-emerging disease, such as influenza and AIDS
  - **Mammalian** – tumorigenic (e.g. MDCK, 293, PER.C6), tumor-derived (e.g. A549, HeLa), tumorigenic + retroviral particles (e.g. CHO)
  - **New species (not previously used)** – avian (e.g. EB66); insect (e.g. Sf9, Hi-Five), plants, bacteria

- Raised safety concerns for known and unknown viruses
- **1st FDA Vaccines and Related Biological Products Advisory Committee (VRBPAC) was in 2001 for transformed, tumorigenic cells (→ 2012 tumor derived cells)**
NGS Capabilities for Broad Virus Detection

2007

Increasing resolution

High Throughput Sequencing

Novel viruses

Emerging viruses

Known viruses

https://str.llnl.gov/april-2013/jaing
Early Public Discussions on New Technologies for Adventitious Virus Detection in Biologics


- **Nov. 2011:** PDA/FDA Adventitious Agents and Novel Cell Substrates: Emerging Technologies and New challenges. Discussed applications and identified knowledge and technical gaps that needed to be address for further development and applications. Conference Proceedings in PDA Journal, 2012

- **Sept. 2012:** FDA VRBPAC discussions on use of human tumor cell lines and potential use of advanced virus detection technologies Transcript pdf available: [www.fda.gov](http://www.fda.gov)

- **May, 2013:** WHO Cell Substrate Study Group (Beijing) discussed DNA based detection of adventitious agents in cell substrates

Challenges for Using NGS for Virus Detection in Biologics

- **Standardization and Validation**
  - Appropriate model viruses and other relevant reference materials/standards *(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
NGS Potential for Broad Adventitious Virus Detection

- NGS discovery of **known and novel viruses** in biological materials *that were missed by previous testing using recommended routine assays*
  - Porcine circovirus type 1 (PCV1) in a licensed rotavirus vaccine (*Victoria et al., 2010*).
  - A novel rhabdovirus in the Sf9 insect cell line used for baculovirus-expressed products (*Ma et al., 2014*).
FDA Efforts on NGS for Applications in Biologics

FDA

- Establishment of FDA and CBER Genomic WGs to support a research and regulatory infrastructure to support policy development and decision-making related to applications of NGS
- Strengthen in-house laboratory and bioinformatics expertise for NGS analysis
- Co-organization of scientific, public meetings focused on data presentations and discussions of NGS readiness for adventitious virus detection in biologics

AVDTIG “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
NGS for adventitious virus detection in biologics with focus on applications for human vaccines and lessons learnt from veterinary vaccines.

The meeting included data presentations and discussions for developing a scientific consensus for using NGS for virus detection in selected applications of biologics.
Report of the 2017 international conference on next generation sequencing for adventitious virus detection in biologicals

Arifa S. Khan a,*, Luca Benetti b, Johannes Blumel c, Dieter Deforce d, William Egan e, Ivana Knezevic f, Philip R. Krause g, Laurent Mallet g, Dietmar Mayer h, Philip Minor i, Pieter Neels j, Guanhua Wang k
2017 Meeting Outcomes

• Participants identified needs for:
  o Standard reference reagents
  o Well-annotated databases
  o Large data storage and transfer capacity
  o Clear and simple strategy for follow up of NGS hits
    o Personnel with relevant expertise, particularly in bioinformatics
    o Harmonization of international regulations for testing biologic products and reagents used for their manufacturing.

• It was noted that continued collaborative efforts and scientific exchange by regulatory and other government agencies, industry, academic labs, and service providers will move the NGS field forward with the goal of assuring the safety of biological products that impact on human and animal health.
AVDTIG Subgroups (2019)

**Subgroup A/B. Co-leaders: Siemon.Ng@sanofipasteur.com and Jean-Pol.Cassart@gsk.com**
- Sample selection/Preparation/Processing
- Reference materials and Virus standards

**Subgroup C. Co-leaders: Arifa.Khan@fda.hhs.gov and Stephane.Cruveiller@pathoquest.com**
- Databases evaluations
- Development of a complete and correctly annotated, publically available virus reference database

**Subgroup D/E. Co-leaders: Christophe.G.Lambert@gsk.com and Robert.Charlebois@sanofipasteur.com**
- Bioinformatics pipelines analysis
- Follow-up strategies to confirm an NGS “hit”
SUBGROUP A

Received: 11 August 2018; Accepted: 11 October 2018; Published: 16 October 2018

Perspective
Current Perspectives on High-Throughput Sequencing (HTS) for Adventitious Virus Detection: Upstream Sample Processing and Library Preparation

Siemon H. Ng 1,*, Cassandra Braxton 2, Marc Eloit 3,4, Szi Fei Feng 5, Romain Fragnoud 6, Laurent Mallet 7, Edward T. Mee 8, Sarmita Sathiamoorthy 1,4, Olivier Vandeputte 9 and Arifa S. Khan 10

SUBGROUP D

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Perspective
Considerations for Optimization of High-Throughput Sequencing Bioinformatics Pipelines for Virus Detection

Christophe Lambert 1,*, Cassandra Braxton 2, Robert L. Charlebois 3, Avisek Deyati 1, Paul Duncan 4, Fabio La Neve 5, Heather D. Malicki 6, Sebastien Ribrioux 7, Daniel K. Rozelle 8, Brandye Michaels 9, Wenping Sun 6, Zhihui Yang 10 and Arifa S. Khan 11
Khan Lab Efforts on NGS Standardization for Adventitious Virus Detection in Biological Products

**Reference Materials**
- Production of Reference Materials (virus stocks, cell-based)
- Characterization
- Storage/Distribution

**Assay Standardization**
- Sample Preparation
- LOD (Spiking Studies)
- Sequencing platforms
- Bioinformatics

**Viral Sequence Database**
- Reference Viral DataBase (RVDB)
- Annotation for Virus-specific Detection
- Database Refinement

**AV Detection Workflows/Pipelines**
- Evaluation of Workflow Performance
- Optimization of Bioinformatic Workflows
Viruses for the study were selected based upon different physicochemical properties, representation of potential viruses of concern in biologics, and commercial availability.

Comparable virus detection was obtained using 4 model viruses spiked in complex matrices by the three laboratories regardless of sample processing, library preparation, sequencing platform, and bioinformatic analysis.
Availability of Reference Virus Stocks for NGS Evaluation and Standardization

<table>
<thead>
<tr>
<th>Virus Name</th>
<th>Total vials prepared*</th>
</tr>
</thead>
<tbody>
<tr>
<td>PCV-1</td>
<td>392</td>
</tr>
<tr>
<td>REO</td>
<td>403</td>
</tr>
<tr>
<td>FeLV</td>
<td>503</td>
</tr>
<tr>
<td>RSV</td>
<td>388</td>
</tr>
<tr>
<td>EBV</td>
<td>490</td>
</tr>
</tbody>
</table>

*volume about 0.5 ml

- Distributed for spiking studies in the AVDTIG

- Available through an FDA MTA: arifa.khan@fda.hhs.gov
Potential Use of Reference Virus Stocks for NGS Standardization

- Perform spiking studies to evaluate total NGS workflow for virus detection in different matrices relevant for biological materials in production of biologics
- Compare NGS with current nucleic acid-based or infectivity assays for virus detection (PCR, *in vivo*, *in vitro*)
- Generate well-characterized datasets for evaluating bioinformatics pipelines
RVDB was found to be more sensitive and specific for virus detection and is expected to enhance HTS investigations for product safety by increasing efficiency of virus detection, particularly for novel viruses. The reduced nonspecific cellular hits resulting in less data volume for bioinformatics analysis and less computational time.

Unclustered (U-RVDB) and Clustered (C-RVDB) have been available at the George Washington University HIVE since Jan. 2017. Proteic versions of RVDB are provided by Marc Eloit and Thomas Bigot (Institut Pasteur; Bigot et., F1000Research, 8:530, 2019).

RVDB has moved to University of Delaware (Oct, 2019)! -> User-friendly, blast-searchable!
RVDB Current Status

- [https://rvdb.dbi.udel.edu/](https://rvdb.dbi.udel.edu/)
- Continue to be updated quarterly and coincident with a GenBank update
- Annotation efforts initiated to characterize some viral entries that also contain non-viral sequences

**RVDBv17.0 and 1st Annotation File (v.16.0)**

- Available since Oct. 29, 2019
  - Number of sequences (U-RVDB/C-RVDB): 2,820,860 / 717,145.
  - Proteic versions are available at RVDB and [https://rvdb-prot.pasteur.fr/](https://rvdb-prot.pasteur.fr/)
RVDB Can Enhance NGS Bioinformatics for Adventitious Virus Detection

- RVDB is aimed to have a complete representation of diverse virus families, specifically from host species used in cell substrates for production of biologics and accurately annotated entries indicating viral/nonviral regions
  - Provide rapid and accurate results for adventitious virus detection
  - Availability of unclustered and clustered nucleotidic RVDBs and corresponding proteic versions can facilitate detection of known and distantly related, novel viruses
  - Development of strategies for NGS large data analysis for adventitious virus detection can facilitate general use of NGS for broader applications in biologics

- Regular updating with new entries deposited in Genbank will aid in detection of emerging viruses
Standards for NGS Detection of Viral Adventitious Agents in Biologics and Biomanufacturing

September 18-19, 2019
NIST Campus
Gaithersburg, Maryland USA

- Currently used reference materials or standards
- What are the appropriate standards for various stages of the sequencing process (except bioinformatics)
- Limitations of current standards
- Current industry practices
- What additional types of standards are needed

CO-CHAIRS: Megan.Cleveland@nist.gov and Arifa.Khan@fda.hhs.gov
Types of Standards for NGS Virus Detection

Natural
- Viral particles
- Extracted viral DNA/RNA

Synthetic
- Virus-like particles
- Naked nucleic acid (ie plasmid)
Different Standards at Different Steps in NGS Workflow

1. Sample collection
2. Reduction of host cell DNA/RNA
3. DNA/RNA Extraction
4. RNA -> cDNA conversion
5. Library Preparation
6. Sequencing
7. Bioinformatic Analysis

Meeting Summary in Preparation
➢ 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.

**Scientific Committee**

- **Dieter Deforce**, Ghent University / Federal Agency for Medicines and Health Products of Belgium (FAMHP)
- **Sebastiaan Theuns**, Ghent University / PROVAXS
- **Arifa Khan**, U.S. Food and Drug Administration (FDA)
- **Pieter Neels**, International Alliance for Biological Standardization (IABS)
- **Sven Arnouts**, Ghent University / PROVAXS
- **Johannes Blümel**, Paul-Ehrlich Institut (PEI)
- **William Egan**, GlaxoSmithKline Vaccines
- **Carmen Jungbäck**, International Alliance for Biological Standardization (IABS)
- **Ivana Knezevic**, World Heath Organization (WHO)
- **Laurent Mallet**, Sanofi Pasteur
- **Gerald Schumann**, Paul-Ehrlich Institut (PEI)
- **David Mackay**, Advisor Veterinary Vaccinology
- **Joseph Victoria**, Boehringer-Ingelheim
What is the readiness of NGS to currently supplement and/or replace assays?

In which situations can NGS be used to supplement or replace current assays? Which assays?

Is there a difference in the expectations for NGS data for supplementing or replacing assays?

What is needed for broader (routine) applications of NGS

Can different regulatory authorities develop same expectations (since we are still in the early phases)?
NGS Workflow for Adventitious Virus Detection is Complex

(PRE-TREATMENT)
- Reduction of “free” nucleic acid using nuclease
- Enrichment of particles by WGA/filtration/size selection/ultracentrifugation

NUCLEIC ACID EXTRACTION
- Whole cells
- Cell lysate
- Supernatant (cell-free)

LIBRARY PREPARATION
- rRNA depletion/polyA+ selection
- cDNA synthesis
- Target-specific amplification
- Fragmentation

SEQUENCING
- Short-read
  o Illumina
- Long-read
  o PacBio
  o Nanopore

BIOINFORMATICS
- Assembly programs
- Analysis tools
- Databases
Next Generation Sequencing Platforms are Evolving

2013:
454 Roche FLX+
A Pyrosequencing system
Yield per Run: 0.4-0.7Gb
Reads Length: up to 700bp
Instrumental Time: 5h
Equipment Cost: $500K

2017:
Illumina Platforms
High throughput Sequencing
Yield per Run: 0.5-3Gb
Reads Length: up to 700bp
Instrumental Time: 2h
Equipment Cost: $695K

2018:
Oxford Nanopore MinION
Miniaturised USB Device
Yield per Run: 5Gb
Reads Length: up to 200kbp
Instrumental Time: 1-48h
Equipment Cost: $1K
Potential Applications of NGS for Adventitious Virus Detection in Biologics

- **Strategy to mitigate risk of AV introduction**
  - Raw materials used for cell culture
  - Cell banks
  - Virus seeds

- **Monitor absence of AV during production**
  - Bulk harvest
  - Final product
Currently Recommended Assays for Adventitious Virus Detection

- **General detection assays**
  - *In vivo* assays (adult mice, suckling mice, embryonated hens’ eggs, guinea pigs)
  - *In vitro* cell culture tests in cell lines of 3 species (same as cell substrate, monkey, human)
  - 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, Infectivity)

- **Additional Assays for Novel Cell Substrates** *(CBER-recommended case-by-case)*
  - Extended PCR assays
  - Oncogenicity assays: Tumor-inducing viruses
  - Chemical induction assays: Endogenous retroviruses, latent DNA viruses
Potential Applications of NGS for Virus Detection in Biologics

➢ Replacement/Alternative assay (in vivo and PCR assays)
  • In vivo AV assays – NGS can provide defined sensitivity and breadth of virus detection
    ▪ Reduce use of animals – meet 3R’s objectives
  • PCR assays – NGS can have similar or greater sensitivity than PCR assays
    ▪ Single assay with broader virus detection

➢ 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
    ▪ Potentially supplement in vitro AV assays as a read-out assay to broaden virus detection

➢ Follow-up of a positive result is critical to determine biological relevance and significance (as with any nucleic acid-based assay). NGS data can aid in design of a “custom” assay to determine if signal due to infectious virus
OVRR: Current Status of NGS Applications

- NGS data is currently under review at CBER
  - Use of NGS for product characterization and testing:
    - Adventitious virus testing of Master and Working Virus Seeds, viral harvests
    - Genetic stability of vaccine virus
    - Cell substrate characterization

- NGS data is considered on a case-by-case basis
  - Often complementary to traditional testing performed to characterize products with regards to adventitious agents
  - Efforts are still underway to standardize and validate NGS

- Within OVRR we highly recommend that sponsors request a technical working group discussion related to the use of NGS and their product characterization
  - Non-regulatory meeting to discuss “plans” for use of NGS
  - Reach consensus prior to initiating lengthy, expensive studies
Assay Standardization and/or Validation

- Include relevant controls (*positive and negative*)
- Determine sensitivity and specificity
- Demonstrate precision (*reproducibility and repeatability*)
- Evaluate assay robustness (*change of assay conditions and reagents*)
- Demonstrate 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)
Testing is *One* Component of the Strategies to Mitigate Risk of Adventitious Viruses

- **Risk assessment**- Identity potential sources of virus introduction to develop a comprehensive risk mitigation strategy and testing plan
  - Know the spectrum of infectious viruses that could potentially be in the host species of source materials (naturally-occurring, animal vaccines)
  - Gain cell culture passage history and characterization
  - Examine potential for virus exposure in the supplier’s facilities (*including chemically-derived materials*)

- **Prevention**
  - Use well-characterized cell banks
  - Use certified/tested animal-derived biological materials (e.g. serum, trypsin, antibodies)

- **Process validation**
  - Incorporation robust viral clearance steps during manufacture for viral clearance and purity [reduction of residual cellular materials (DNA, RNA, proteins)]

- **Testing**
  - Extensive testing for known and unknown agents in the starting materials (cell substrate, virus seeds, vector virus preparation)
  - Adventitious agent testing at different stages in manufacturing process and at steps with the greatest potential for contamination
  - Using various improved sensitive and broad detection assays
Introduction of Improved Assays – Path Forward

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

Updating ICH Q5A(R2):
Viral Safety Evaluation of Biotechnology Products Derived from Cell Lines of Human or Animal Origin

➢ The final Concept Paper and Business Plan was endorsed by the ICH Management Committee on Nov 18 2019 (Singapore)

➢ The new guideline is anticipated to take three years to achieve Step 4, from November 2019 – November 2022.
  • Completion of Step 1, Step 2a and 2b: June 2021
  • Completion of Step 3 and 4: November 2022
Updating ICH Q5A(R2):
Viral Safety Evaluation of Biotechnology Products Derived from Cell Lines of Human or Animal Origin (cont.)

• The current guideline was finalized in 1999
• Updates will reflect
  • New classes of biotechnology products
  • Additional validation approaches for virus clearance
  • New virus assays and alternative analytical methods for virus testing
    • nucleic acid-based assays such as Polymerase Chain Reaction (PCR) and Next Generation Sequencing (NGS) may provide rapid and sensitive detection of adventitious and endogenous viruses in the starting and harvest materials.
    • general principles for the inclusion of new assays and potential replacement/supplement of existing assays should be presented in order to continue to support future development of new technology.
• Virus clearance validation and risk mitigation strategies for advanced manufacturing
• Aspects of virus clearance validation that have emerged or evolved
THANK YOU!