FDA Perspective on Using NGS/HTS for Adventitious Virus Testing

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February 22, 2024
Disclaimer

This presentation is an informal communication and represents my own best judgment. The material in this presentation and my comments do not bind or obligate FDA or any other agency.
Viral Factors Influencing Safety and Quality of Biologics

EXTRANEOUS VIRUSES

- Manufacturing-related (all product types)
  - Adventitious viruses – Raw materials (Cell substrate, cell culture reagents), personnel, equipment, facilities
  - Endogenous retroviruses - Cell substrate

- Process-related (viral vectors and viral vector-based products)
  - Replication-competent viruses – de novo generation
  - Helper viruses – used with some viral vectors

VIRAL IMPURITIES

- Product-related
  - Viral nucleic acids from disrupted cells – free RNA or DNA; integrated DNA (e.g., 293, HeLa)
  - Encapsidated “irrelevant” sequences in viral vectors – viral or cellular (e.g., rAAV)
  - Plasmids used in transductions
An Integrated Strategy for Adventitious Virus Risk Mitigation

**PREVENTION**
- **Risk assessment** Identify 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*)
- **Use qualified materials**
  - Well-characterized cell banks
  - Certified/tested animal-derived biological materials (e.g. serum, trypsin, antibodies)

**CLEARANCE (not applicable for all products!)**
- Incorporate robust viral clearance steps during manufacturing to validate the process
  - Viral inactivation and removal
  - Product 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**
Routine Viral Adventitious Agents Tests for Product Safety

- **General virus 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**
  - *In vitro* tests for animal viruses e.g., bovine, porcine (9CFR 113.47 and 113.53)
  - *In vivo* antibody-production assays for rodent viruses (MAP, including LCMV challenge; HAP; RAP)
  - Assays for known viruses (PCR, Infectivity)

- **Additional assays for virus detection**
  - Extended and broader PCR-based assays
  - Expanded cell culture assays (*adding more target cell lines*)
  - Chemical induction assays: Latent viruses (endogenous and episomes)

_The currently recommended assays have been generally effective in demonstrating the absence of adventitious viruses for product safety_
Limitations of Currently Recommended Adventitious Virus Tests

- **Cell-culture assays**
  - Based upon susceptibility of target cells to virus infection
  - Assay read-out is a visible effect due to virus replication, such as cytopathic effect (CPE), hemadsorption, hemagglutination
  - Sample-related interference
  - 28-day observation period

- **Animal-based assays**
  - Unknown sensitivity for virus detection
  - Detection depends on susceptibility of animal species to virus infection
  - Based upon a measurable pathological effect due to a replicating virus
  - Sample-related interference
  - > 18 day-observation period depending upon the species
  - Use of animals globally discouraged (3 R’s initiative!)

- **Molecular assays (PCR)**
  - Designed based upon available known virus sequences
  - Large number of assays needed for detection of different viruses

- **Additional assays: Chemical induction**
  - Can activate latent viruses, but detection of induced, unknown viruses would be missed due to using the conventional methods for virus detection
NGS for Broad Detection of Adventitious Viruses

- NGS was initially recognized as a powerful advanced technology for adventitious virus detection by the identification of PCV1 in a licensed rotavirus vaccine and the subsequent discovery of a novel rhabdovirus in the insect Sf9 cell line (used commonly for baculovirus-expressed vaccines and other biologics).

- In both cases, routine testing had been done. Additional testing using degenerate PCR assays to detect various insect virus families was done for the Sf9 cells since it was a novel cell substrate.

- The testing gaps and complexity of the NGS technologies were recognized in CBER and by industry leading to their combined efforts for establishing the Advanced Virus Detection Technologies Interest Group in the the Paternal Drug Association (PDA).
General Challenges of NGS Applications for Virus Detection

- **Standardization and validation**
  - Appropriate reference viruses and other standards *(for spiking studies)*
    - Efficiency of the different steps involved in the methodology
    - Sensitivity and specificity

- **Bioinformatics**
  - Data analysis
    - Pipeline optimization
      - Reference datasets
      - Criteria for acceptable quality of reads
      - Parameters for short read assembly; hybrid assembly to correct high error-rate currently seen in long-read sequencing
    - Development of a complete and correctly annotated, publicly available, Reference Virus Database
    - Develop strategies for novel virus detection
  - 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
Advanced Virus Detection Technologies IG

**Mission:** To advance the tools for the next generation of viral risk evaluation by providing an informal, scientific forum for discussions and scientific collaborations *(initial focus: HTS)*

-> Through scientific discussions, knowledge exchange, and collaborative studies.

More than 216 participants from over >60 organizations:
- Regulatory agencies
- Government agencies
- Industries
- Service providers
- Technology developers
- Academics

**Co-chairs**
- **Arifa S. Khan** (FDA, U.S.A.; October, 2012)
- **Siemon Ng** (Notch Therapeutics, Canada; June, 2022)
- **Ken Kono** (National Institute of Health, Japan; October, 2023)
- **Noémie Deneyer** (GSK, Belgium; November, 2023)

Meeting/discussions by t-con once every 2 months
AVDTIG – Objectives

- Provide an **informal, scientific forum** for discussions, knowledge exchange, and scientific collaborations among scientists from different organizations
- Develop **consensus views**
- Promote and Conduct IG **collaborative studies**
- Publish **best practices, perspective and position papers** for considerations of NGS applications in biologics
- Interact with other consortiums to **enhance the IG goals and extend efforts**.
AVDTIG Subgroups

2012

Subgroup A
Sample selection/ preparation/processing

Subgroup B
Virus standards and reference materials

Subgroup C
Complete and correctly annotated, virus reference database

Subgroup D
Bioinformatics pipelines analysis

Subgroup E
Follow-up strategies to confirm the identity of a “hit”

2018

Subgroup AB

Subgroup C

Subgroup DE
Achievements and Contributions of AVDTIG
AVDTIG – Subgroup AB: Collaborative Spiking Studies

- For performance evaluation and standardization of NGS
  - Evaluate reference standards and bioinformatics tools
  - Compare and optimize experimental protocols

Spiking study 1
(2013-2016)

- HeLa cell line

- EBV
- RSV
- FeLV
- Reo
- PCV1

Spiking study 2A
Started 2017
- Virus in CHO background
  - (short-read)

Spiking study 2B
Started 2016
- Viral seed/Viral vector
  - background (short-read)

Spiking study 3
Started 2019
- Transcriptomics (short-read)

Spiking study 4
Started 2020
- Viral seed/Viral vector
  - background: 2B
    - (long-read)

Spiking study 5
Discussions- 2023
- Cell substrate/high cellular
  - background (long-read)
Development of Reference Viruses

❖ Characterization

• Infectious titer per mL (>10^6 TCID_{50} per mL)
• Number of particles: TEM
• Genome copy number: ddPCR (>10^8 gc per mL)
• Adventitious virus analysis: Illumina HTS
• Host DNA copy number: ddPCR (different species)
• Reference virus genome sequence and variant analysis: HTS
• Stability studies: infectious titer; genome copy number

➢ Vialiied individually to allow freedom for custom-mixing, as needed by user

○ CBER has supported storage and distribution at ATCC. Transferred to NIH/NIAID BEI Resources Repository from Sept 29, 2023 for long-term storage and distribution
WHO International Reference Reagents for Adventitious Virus Detection in Biological Products Using HTS (Oct. 2020)

- Developed by CBER based on the results of the first AVDTIG spiking study 1.
- Adopted as WHO reagents by ECBS based on results from the AVDITG Spiking Study 2B
- Characterization: Infectious titer, Particle count, Genome copy number and NGS analysis

<table>
<thead>
<tr>
<th>Virus</th>
<th>Genome type</th>
<th>Genome size</th>
<th>Particle size</th>
<th>Envelope</th>
<th>Chemical resistance</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reo (Lang)</td>
<td>RNA, double-strand; Linear (segmented)</td>
<td>23.6kb(1,196–3,915nt)</td>
<td>80nm</td>
<td>No</td>
<td>Medium-high</td>
</tr>
<tr>
<td>FeLV (KT)</td>
<td>RNA; single-strand; Linear (dimeric)</td>
<td>8.5kb</td>
<td>80-100nm</td>
<td>Yes</td>
<td>Low</td>
</tr>
<tr>
<td>RSV (A2)</td>
<td>RNA; single-strand; Linear</td>
<td>15kb</td>
<td>150-200nm</td>
<td>Yes</td>
<td>Low-medium</td>
</tr>
<tr>
<td>PCV-1*</td>
<td>DNA, single-strand; circular</td>
<td>1.8kb</td>
<td>16-18nm</td>
<td>No</td>
<td>High</td>
</tr>
<tr>
<td>EBV (B95-8)**</td>
<td>DNA, double-strand; Linear</td>
<td>172kb</td>
<td>122-180nm</td>
<td>Yes</td>
<td>Low-medium</td>
</tr>
</tbody>
</table>

* Also contains Porcine Endogenous Virus
** Also contains Squirrel Monkey Retrovirus

- Request for the 5 model viruses can be made via the NIAID BEI Repository catalogue number NR-59622
- Information regarding the virus stocks can be made by contacting CBER, Arifa.Khan@fda.hhs.gov
## Proposed WHO Reference Virus Panel for NGS/HTS Adventitious Virus Detection

<table>
<thead>
<tr>
<th>WHO Intl Ref Reagents – Currently Available</th>
<th>Proposed 1st WHO Intl Ref Virus Panel – Q1-2/2024</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Particle size (nm)</strong></td>
<td><strong>Envelope</strong></td>
</tr>
<tr>
<td>Epstein-Barr virus type 1</td>
<td>122-180</td>
</tr>
<tr>
<td>Feline leukemia virus</td>
<td>80-100</td>
</tr>
<tr>
<td>Human respiratory syncytial virus type A</td>
<td>150-300</td>
</tr>
<tr>
<td>Human reovirus type 1</td>
<td>60-80</td>
</tr>
<tr>
<td>Porcine circovirus type 1</td>
<td>16-18</td>
</tr>
<tr>
<td>Human coronavirus HCoV-OC43</td>
<td>80-120</td>
</tr>
<tr>
<td>Minute Virus of Mice</td>
<td>26</td>
</tr>
</tbody>
</table>

**Virus Types:*** Herpesvirus, Retrovirus, Paramyxovirus, Reovirus, Circovirus, Coronavirus, Parovirus
A Comprehensive Reference Virus Database (RVDB) for NGS Broad Virus Detection

➢ To address the deficiencies in the public databases, we developed a new reference virus database based upon semantic selection from GenBank and NCBI RefSeq + Neighbor Genomes (Goodacre et al., mSphere, 2018)
  ▪ Contained all viral sequences regardless of size
  ▪ Included endogenous viral and retroelements
  ▪ Has a reduced cellular content
  ▪ The latest version RVDBv28.0 (Nov 22, 2023) is available at https://rvdb.dbi.udel.edu/ with link for proteic RVDBs generated by Marc Eloit and Thomas Bigot (http://rvdb-prot.pasteur.fr/).

➢ Provides high diversity of viral sequences to increase likelihood of novel virus detection, with reduced nonspecific cellular hits resulting in less data volume for bioinformatics analysis (and less computational time!)
➢ Updated quarterly by the Khan Lab (Pei-Ju Chin)
➢ Ongoing work on annotation of sequences to remove misannotated sequences and enhance virus-specific detection (Pei-Ju Chin & Trent Bosma)
RVDB Provides 4 Formats to Adapt Various Application Scenarios

- **U-RVDB fasta file**
  - Un-clustered, contain all viral sequences with redundancy
  - Higher computation-demanded. **Suitable for virus detection by blastn/nhmmmer**

- **C-RVDB fasta file**
  - Clustered, sequences share 98% similarity are collapse to one representative sequence for each clade
  - Lower computation-demanded. **Suitable for virus detection by tblastx**

- **SQLite DB Script**
  - Create the entries (*fasta* header and the corresponding information) for advanced bioinformatic pipelines/workflows

- **Proteic RVDB (provided by Institut Pasteur: https://rvdb-prot.pasteur.fr/)**
  - **Hidden Markov Model (HMM)** profile of viral protein domains
  - Unknown viruses with remote homology by *hmmsearch*/*hmmscan*
The Changing Landscape of NGS Applications in OVRR: COVID-19 Era

- OVRR has been receiving submissions requesting use of NGS as a broad adventitious virus test (pre-COVID).
- The number of requests have increased in 2020 for using NGS an alternative adventitious virus detection assay to accelerate SARS-CoV-2 vaccine development.
  - Increase in the number of sponsors using NGS
    - Increased in-house capabilities and commercial availability
  - Expanded use of NGS for product characterization and testing
    - Cell substrate characterization
    - Testing of Master and Working Virus Seeds and DS Harvest
    - Genetic stability of vaccine virus
  - Extended use of NGS for a virus detection
    - Complementary or supplementary assay -> Replacement of one or more conventional virus detection assays
## CBER/OVRR: Examples of Submissions Using NGS for Adventitious Virus Detection

<table>
<thead>
<tr>
<th>Sample Type</th>
<th>NGS Application</th>
<th>Role of NGS</th>
<th>Outcome</th>
</tr>
</thead>
<tbody>
<tr>
<td>2009- Novel CS- EOP cells and supernatant</td>
<td>To broaden detection of unexpected and unknown/novel viruses</td>
<td>Complementary – Additional; Full conventional testing done</td>
<td>2013-Results used for supporting submission</td>
</tr>
<tr>
<td>2011- Novel CS-WCB, EOPC</td>
<td>To broaden detection of unexpected and unknown/novel viruses</td>
<td>Complementary – Additional; Full conventional testing done</td>
<td>2015- Re-analysis of data with updated database</td>
</tr>
<tr>
<td>2019- MVS, Bulk Harvest</td>
<td>To potentially replace PCR assays</td>
<td>Supplementary – to fill gaps in vitro AV assays due to assay interference</td>
<td>Results as FYI.</td>
</tr>
<tr>
<td>2020- 2022 &gt; 25; Cell Banks, MVS, WVS, Bulk Harvest</td>
<td>To replace <em>in vivo</em> or/and <em>in vitro</em> assays</td>
<td>Complementary, Supplementary, or Replacement due to assay interference</td>
<td>Method validation, Assay qualification/validation</td>
</tr>
<tr>
<td>2023 – *Live, viral vaccine, License: MVS, Bulk DS</td>
<td>To replace <em>in vivo</em> adventitious virus assays</td>
<td>NGS for Replacement of the <em>in vivo</em> adv virus testing due to challenges of the <em>in vivo</em> assays</td>
<td>Performed acceptable assay qualification and validation</td>
</tr>
</tbody>
</table>
NGS Applications in the FDA

NGS data is currently under review at CBER

- Adventitious virus testing of Cell Banks, Virus Seeds, and Bulk Harvests
- Genetic stability of vaccine virus
- Cell substrate characterization

- The CBER Advanced Technologies Team (CATT) coordinates scientific discussions on new technologies including NGS

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

- NGS for AV testing is being applied in gene therapies (CBER/OTP)

- The Emerging Technologies Team in CDER is involved in discussions on NGS for adventitious virus detection in biotherapeutics

- Interest in considering HTS for AV detection has also been initiated for animal-based biologics (CVM)
The update reflects current scientific knowledge and biotechnology advances related to:

- **New classes of biotechnology products** *(amenable to virus clearance)* e.g., baculovirus-expressed VLPs and proteins; AAV vectors; helper-dependent [adenovirus, HSV] viral vector products *(ANNEX 6: Genetically-engineered viral vectors and viral vector-derived products)*
- Additional validation approaches for virus clearance e.g., modular validation
- **New virus assays and alternative analytical methods** e.g., PCR and **NGS/HTS** *(3.2 Recommended Virus Detection and Identification Assays 3.2.5 Molecular Methods)*
- Virus clearance validation and risk mitigation strategies for advanced manufacturing (e.g. continuous manufacturing)
- Aspects of virus clearance validation that have emerged or evolved
ICH Q5A(R2): New Nucleic Acid-Based Test Methods

- Guideline encourages use of new alternative tests (includes Next Generation Sequencing and PCR in discussion) -> **Aligns with the 3Rs initiative to reduce animals for testing**

- Specific opportunities to replace existing methods with targeted test (e.g. PCR or NGS) for replacing antibody production tests or non-targeted (agnostic/broad; such as NGS) for replacing *in vivo*/*in vitro*
  
  - Antibody Production Tests in Rodents (MAP, HAP, RAP)
  
  - *In Vivo* Assays
  
  - *In Vitro* Assay

  ➢ Highlights that direct head-to-head comparison with existing methods is generally not expected (*in vivo and in vitro*)

It is recognized that these nucleic acid-based assays have limitations as they cannot distinguish between infectious and noninfectious particles and therefore detection of a signal may need a confirmatory test with an infectivity assay for risk-assessment.
General Acceptance of NGS for Adventitious Virus Detection in Biologics

- **Cell substrate characterization** – particularly for novel cell substrates or in case where there are concerns for occult and novel viruses

- **Supplementary to or replacement of 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

- **Replacement assay (*in vivo* and PCR assays)**
  - *In vivo* AV assays – HTS can provide defined sensitivity and breadth of virus detection
  - Antibody Production Assays – MAP, RAP, HAP
    - *Reduce use of animals – meet the global objectives for 3Rs (reduction, refinement, replacement)*
  - PCR assays – HTS can have similar sensitivity to PCR assays
    - Single assay with broader virus detection

- As with any assay, implementation of NGS needs method qualification and validation
Current Thinking on Using NGS as an Alternative Adventitious Virus Detection Method

➢ **Replacement assay** (*in vivo* assays; PCR assays)
  • *In vivo* AV assays – NGS can provide defined sensitivity and breadth of virus detection
  • PCR assays – NGS can have similar sensitivity than PCR assays; broader virus detection; single assay

➢ **Supplementary or Replacement 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

❖ **Note**
  • *Follow-up of a positive result is critical to determine biological significance of a signal for decision-making (as for any nucleic acid-based detection assay)*
  • *NGS data may help design a “custom” assay to determine if signal due to infectious virus*
Follow-Up of NGS Signal

Follow-up of a positive result is critical

- Verification of results
  - Can the results be confirmed by PCR or another assay?
  - Is a complete viral genome present?
  - Are particles present?
  - Are the particles infectious?
  - Is there a replication-competent virus?
  - Can the nucleic acid/particles be quantified?

- Determine biological relevance and significance (as with any nucleic acid-based assay)

- NGS data can aid in design of a “custom” infectivity assay for risk management
Potential Applications of NGS for Safety and Characterization of Biologics

- Testing to mitigate risk of adventitious virus introduction
  - Raw materials for cell culture
  - Cell banks
  - Virus seeds

- Monitoring absence of extraneous viruses during production
  - Bulk harvest
  - Final product

- Detection/Characterization of extraneous viral sequences in the final product
  - Encapsidated extraneous sequences in viral vectors
  - Genome analysis

- Characterization of viral vector sequences
  - Identity testing
  - Persistence, Expression
  - Vector integration site analysis
Introducing NGS for Improving Viral Safety Testing

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

- Current cell substrate and viral safety guidances and regulatory documents provide flexibility for using alternative approaches with broad virus detection capabilities and “fit-for-purpose”
  - US FDA (2010)
  - WHO (2010, pub. 2013)
  - ICH Q5A(R2) (2023)
  - Ph. Eur. (2.6.41, public comments April 2023)
Still More Work for Routine Implementation!

• Optimization of pre-treatment conditions to increase sensitivity of virus detection in complex matrices
• Development of SOPs and test datasets for establishing NGS by an early user of the technology
• Development of other types of standard materials (e.g., VLPs)
Collaborative Study- NIIMBL/GHF

- Study participants: FDA/CBER, GSK, Millipore Sigma (BioReliance and EMD Serono)
- A head-to-head study for evaluating NGS with *in vivo* and *in vitro* adventitious virus detection assays (*using same sample material*)
- Two model viruses from the WHO reference virus reagents were used to spike a complex matrix representing a bioreactor sample for protein production.
- Results are in preparation for publication
  - facilitate data-based, decision-making for NGS as an alternative method to replace *in vivo* assays and to replace or supplement *in vitro* assays
Ongoing Work on Reference Materials (Khan Lab)

• Development of a viral reference reagent for cell-based biologics
  • Human cell line with stably integrated retrovirus (cell clones with latent and active infection)
  • Well-characterized for viral genome copy number and integration site(s)
  • Can be used for spiking into an uninfected cell background to evaluate sensitivity of virus detection by NGS in cell substrates and cell-based products
Acknowledgements

- KHAN LAB*
  - Current
    - Pei-Ju Chin
    - Trent Bosma
    - Sandra Fuentes
    - Hailun Ma
    - Nicholas Mattson
    - Andrea Kennard
  - Past
    - Jen-Hui Tsou
    - Norman Goodacre
    - Subhiksha Nandakumar
    - Aisha Aljanahi
    - Syed Shaheduzzaman

- Univ. of Delaware
  - Shawn Polson
  - Karol Miaskiewicz
  - Jaysheel Bhavsar
  - Madolyn Macdonald
  - Kelvin Lee

- Advanced Virus Detection Technologies Interest Group (AVDTIG)
  - Subgroup Leaders & Spiking Study Members
    - Fabio La Neve and Davide Scaglione-MerckGroup (NGS of original 5 virus stocks)

- FDA/CDRH (HPC Team)
  - Mike Mikailov and Fu-Jyh Luo

- FDA/CBER (HTS Core, Rongfong Shen)
  - Jenie Phue, Wells Wu, San-eun Lee

*Funding: FDA Medical Countermeasures Initiative; CBER Targeted Intramural Research – PDUFA; CBER COVID-19 funds; DVP funding; NIIMBL-GHF: CRADA
Thank You!