Control of Viral Safety for Medicinal Products by Deep Sequencing Technology

Keisuke Yusa
Kobe University
COI Disclosure

Keisuke Yusa, Kobe University

I have no potential conflicts of interest in relation to this presentation
1. Conventional tests for endogenous viruses and adventitious viruses

2. Endogenous retrovirus generated from CHO cells

3. Fetal bovine serum as raw material and its safety

4. Virus testing by deep sequencing technology (NGS, HTS, MPS)
<table>
<thead>
<tr>
<th>Virus</th>
<th>Substrate</th>
<th>Source</th>
<th>Product or Company</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>MVM (Minute Virus of Mice)</td>
<td>BHK cells</td>
<td>Contaminated FCS</td>
<td>Wellcome Foot-and-Mouth Disease Vaccine Laboratory</td>
<td>Nettleton &amp; Rweyemamu, 1980</td>
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<td>Bluetongue Virus</td>
<td>Undisclosed</td>
<td>Unknown</td>
<td>Multivalent modified live vaccine against canine distemper virus, adenovirus, and parvovirus vaccines</td>
<td>Akita et al., 1994</td>
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<td>EHDV (Epizootic Hemorrhagic Disease Virus)</td>
<td>CHO Cells</td>
<td>Contaminated raw material</td>
<td>Bioferon GmbH &amp; Co. Recombinant protein for phase I clinical trials</td>
<td>Burstyn, 1996</td>
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<td>MVM</td>
<td>CHO Cells</td>
<td>Contaminated raw material</td>
<td>Pulmozyme®, Genentech</td>
<td>Garnick, 1996</td>
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<tr>
<td>Vesivirus 2117</td>
<td>CHO cells</td>
<td>Contaminated FBS</td>
<td>Boehringer Ingelheim Pharmaceuticals</td>
<td>Oehmig et al., 2003</td>
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<td>Reovirus</td>
<td>Unprocessed bulk harvest (CHO cells)</td>
<td>Contaminated FBS</td>
<td>Undisclosed</td>
<td>Nims et al., 2006</td>
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<td>CVV (Cache Valley Virus)</td>
<td>CHO cells</td>
<td>Contaminated FBS</td>
<td>Undisclosed</td>
<td>Nims et al., 2008</td>
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<tr>
<td>Vesivirus 2117</td>
<td>CHO cells</td>
<td>Contaminated raw material</td>
<td>Cerezyme® and Fabrazyme®, Genzyme</td>
<td>Genzyme Corporation, 2009</td>
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<td>Porcine circovirus</td>
<td></td>
<td></td>
<td>rotavirus vaccine</td>
<td>Gilliland et al., 2012</td>
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</tbody>
</table>

Viral Contamination Related to Cell Lines and Human and Animal Biologics
Risk of Virus Contamination for Biotechnological Products

**Construction of cell bank**
- Transfection of the expression plasmid
- Selection of clones
- Cell bank

- Virus-infected cells are used to generate cell banks.
- Virus is used for establishment of cell lines.
- Cell bank inherently produce endogenous virus like particles.
- Cells are contaminated through raw materials used for cell culture.
- Cells are contaminated with adventitious viruses when manipulating.

**Cell Culture**
- WCB (frozen vial)
- Culture volume expansion
- Production culture
- Culture fluid

- Cells are contaminated through raw materials.
- Cells are contaminated with adventitious viruses when manipulating.

**Purification process**
- Unprocessed bulk (centrifugation/depth filtration)
- Chromatographic purification
  - Low pH treatment
  - Virus filtration
- Drug substance

- Purification substrate is contaminated with virus.
- Intermediate is contaminated with virus when manipulating

**Virus tests for cell bank**

**Testing for viruses in unprocessed bulk**

**Evaluation and characterization of viral clearance**
Cell Bank System

• In order to use the same material for all of production, cell substrate should be frozen and stored as cell bank. → MCB (Master Cell Bank)
• WCB (Working Cell Bank) should be prepared from MCB. The cell bank system ensures a stable quality of products.

➢ Cell bank is a common substrate for biopharmaceuticals, vaccines, gene therapeutic products, and cell based products,
➢ For viral safety of the final products, virus testing of cell bank is indispensable.
Virus Testing for Cell bank

MCB
- Tests for retroviruses
- In vitro assay
- In vivo assay
- Antibody Production tests
- Cell specific virus tests

WCB/CAL
- Tests for retroviruses
- In vitro assay
- In vivo assay

Transfection/cloning/amplification and stock

MCB

WCB

Thawing & expansion

Purification process

CAL (Cells at the limit of in vitro cell age used for production)
## Virus testing for Cell Bank

<table>
<thead>
<tr>
<th>Test</th>
<th>Details</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>In vitro assay</strong></td>
<td>• CPE (cytopathic effect) observation of MRC-5 cells with cell lysate</td>
</tr>
<tr>
<td></td>
<td>• CPE (cytopathic effect) observation of Vero cells with cell lysate</td>
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<tr>
<td></td>
<td>(hemagglutination (HA) / adsorption test at endpoint)</td>
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<td></td>
<td>• Extended XC plaque assay using SC-1 cells (Ecotropic mouse retrovirus test)</td>
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<td></td>
<td>• Observation of cells with transmission electron microscope to detect presence of retrovirus particles</td>
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<td></td>
<td>• Reverse transcriptase activity in cell lysate</td>
</tr>
<tr>
<td><strong>In vivo assay</strong></td>
<td>• Adult mice inoculated with cell lysate and collect organs in 28 days</td>
</tr>
<tr>
<td></td>
<td>• Suckling mice inoculated with cell lysate in 14 days, and the cell lysate is inoculated in adult mice in 28 days</td>
</tr>
<tr>
<td></td>
<td>• Allantoic cavity of chick embryonated eggs inoculated with cell lysate, and HA test (chicken, guinea pig, human type O erythrocytes). Inoculation of cell lysate into yolk sac of eggs and observed for 10 days</td>
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<tr>
<td><strong>Retrovirus assay</strong></td>
<td>• S+L- Focus assay using mink S+L- cells (Xenotropic mouse retrovirus test)</td>
</tr>
<tr>
<td></td>
<td>• Extended XC plaque assay using SC-1 cells (Ecotropic mouse retrovirus test)</td>
</tr>
<tr>
<td><strong>Tests for viruses derived from bovine</strong></td>
<td>• CPE (cytopathic effect) observation of Bovine turbinate cells with cell lysate for 14 days.</td>
</tr>
</tbody>
</table>

Cost: >$64,000–$73,000 ($7–8,000,000 yen) >2 month
Current Virus Testing

• ICH Q5A provides the design of viral tests and experiments for the assessment of viral clearance.
• The conventional virus tests require time, high cost and BSL2 facilities/technical experts to use alive viruses.
• A virus detection window of each assay is restricted.
Agenda

1. Conventional tests for endogenous viruses and adventitious viruses
2. Endogenous retrovirus generated from CHO cells
3. Fetal bovine serum as raw material and its safety
4. Virus testing by deep sequencing technology (NGS, HTS, MPS)
Endogenous Retrovirus

CHO cell derivatives have been broadly used as cell substrate due to their advantages for production of recombinant proteins, however, they constitutively produce retrovirus-like particles (RVLPs).
## RVLPs Generated from CHO and Murine Hybridoma

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<thead>
<tr>
<th></th>
<th>CHO</th>
<th>murine hybridoma</th>
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</thead>
<tbody>
<tr>
<td><strong>Localization</strong></td>
<td>cytoplasm</td>
<td>intracellular vesicles</td>
</tr>
<tr>
<td><strong>Cell culture fluid</strong></td>
<td>$&lt; 10^3$-$10^6$ /mL</td>
<td>$\sim 10^6$-$10^8$ /mL</td>
</tr>
<tr>
<td><strong>Infectivity</strong></td>
<td>not infectious</td>
<td>positive in S+L- focus assay/ not infectious to human and primate cells</td>
</tr>
</tbody>
</table>

Endogenous Retrovirus Genes Have Been Isolated from CHO Cells but They Were not Responsible for RVLP Production

<table>
<thead>
<tr>
<th>Sequence</th>
<th>Similarities &amp; size</th>
<th>Genes</th>
<th>Library and Cloning Probe</th>
<th>ERV RNA</th>
<th>references</th>
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</thead>
<tbody>
<tr>
<td>CHIAP.ML10</td>
<td>0.95 kb</td>
<td>pol (RNase)</td>
<td>CHO VLP cDNA Library MLV probe</td>
<td>+</td>
<td>Anderson et al. (1991) J.Virol. 181: 311</td>
</tr>
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<tr>
<td>CHIAP I *</td>
<td>SW2 (68%) , 1.9 kb</td>
<td>p27-Δ-env</td>
<td>CHO cDNA library with Sirian hamster IAP probe</td>
<td>+</td>
<td>Anderson et al. (1990) J.Virol. 64: 2021-2032</td>
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<tr>
<td></td>
<td>YL6 (69%) , 1.6 kb</td>
<td>p27-Δ-env</td>
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<td>Anderson et al. (1991) Dev. Biol. Stand. 75:123-132</td>
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<tr>
<td>CHIAP II *</td>
<td>YL7 (54%) , 2.2 kb</td>
<td>p12-pro</td>
<td>CHO cDNA library with Sirian hamster IAP probe</td>
<td>+</td>
<td>Anderson et al. (1991) Dev. Biol. Stand. 75:123-132</td>
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<td></td>
<td>YL9 (59%) , 2.2 kb</td>
<td>pro-rt</td>
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<td>CHIAP34</td>
<td>6.4 kb gag, pro-pol, env</td>
<td>gag (1.6 kb ORF)-pol (1.1 kb ORF)-env (-)</td>
<td>CHO genomic library SHIAP (H18, pol) probe</td>
<td>-</td>
<td>Dorner et al. J. Virol. (1991) 65: 4713-4719</td>
</tr>
</tbody>
</table>
CHO Cells Generate 3 RVLPs: CHERV-1b, CHERV-2g, CHERV-3g

- >565 ERV copies in C. griseus genome estimated by LTRharvest
- 62 ERV transcripts containing gag like seq in C. griseus mRNA database
- 5 of 62 ERV polypeptides from gag ORF
- 3 of 5 ERV RNA identified in VLPs
- CHERV-1b
- CHERV-2g
- CHERV-3g
Phylogenetic Tree of CHO RVLP Genes

**Betaretrovirus**

**Gammaretrovirus**

Yuan et al., *unpublished data*
Endogenous Retroviruses from CHO K-1 Cells

• We found that three RVLP genes are still active to generate RVLPs, and designated as CHERV-1b, CHERV-2g, and CHERV-3g.
• RVLP gene knockout CHO should be safer substrate for manufacturing recombinant proteins and antibodies.
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FBS Is Highly Contaminated with Bovine Virus Nucleic Acids

- BVDV is a highly prevalent infection of cattle and its presence in bovine serum cannot completely be avoided except in serum from specifically controlled donor herds or from cattle from BVDV-free geographic areas.

- Bovine polyoma virus (BPyV) is a common contaminant of bovine serum.

Virus genomes are detected in 16 lots of fetal bovine serum

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<th>Virus Name</th>
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<th>GTM</th>
<th>FRA</th>
<th>IRL</th>
<th>FRA</th>
<th>FRA</th>
<th>AUS</th>
<th>USA</th>
<th>unknown</th>
<th>USA</th>
<th>USA</th>
<th>CHL</th>
<th>CHL</th>
<th>DMA</th>
<th>FRA</th>
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<td>BRSV (bovine RS virus)</td>
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<td>BTV (bluetongue virus)</td>
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<td>BVDV1 (Bovine viral diarrhea virus 1)</td>
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<td>BVDV2 (Bovine viral diarrhea virus 2)</td>
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<td>BPV1 (bovine papillomavirus)</td>
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<td>Bylma (bovine polyomavirus)</td>
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<td>CVV (Cache valley virus)</td>
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</tbody>
</table>

Virus nucleotides were detected by sequencing or nested PCR.

Yuan et al., *unpublished data*
Virus Genomes Are Detected in 16 FBS Lots

Yuan et al., *unpublished data*
Viral Safety on Bovine Serum

Bovine serum should be tested based on FDA 9CFR113.53 or EMA/CHMP/BWP/457920/2012 rev.1, and gamma-irradiated for minimizing the risk of virus contamination.

1. Title 9 of the Code of Federal Regulations(CFR 113.53 Requirements for ingredients of animal origin used for production of biologics.
Agenda

1. Conventional tests for endogenous viruses and adventitious viruses
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New Assay Technologies for Viral Safety Are Described in Q5A

• The conventional assays described in ICH Q5A should be regarded as assay protocols recommended, but not all-inclusive or definitive.

• Since the most appropriate techniques may change with scientific progress, proposals for alternative techniques may be acceptable, when accompanied by adequate supporting data.

• Manufacturers are encouraged to discuss these alternatives with the regulatory authorities.
Q.

NGS is expected to provide a rapid, sensitive and comprehensive test to detect virus nucleic acids. Can we replace certain part of conventional virus tests with an alternative test using NGS?
Targets of Virus Nucleic Acids by NGS

Types and forms of nucleic acids contained in virus particles
Targets for Detection of Viruses in Infected Cells

- **viral DNA**
- **vRNA or vDNA**
- **PCR**
- **RT-PCR**
- **WB**
- **ELISA**
- **TEM**

### Method

<table>
<thead>
<tr>
<th>Target</th>
<th>viral DNA</th>
<th>viral RNA</th>
<th>virus polypeptide</th>
<th>virus shape</th>
<th>Virus particle, vRNA or vDNA</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Method</strong></td>
<td><strong>PCR</strong></td>
<td><strong>RT-PCR</strong></td>
<td>• RT activity</td>
<td>• TEM</td>
<td>• PCR, • RT-PCR, • TEM</td>
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<tr>
<td>RNA virus</td>
<td>ND</td>
<td>ND</td>
<td>• WB</td>
<td></td>
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<tr>
<td>ND latent virus</td>
<td></td>
<td></td>
<td>• ELISA</td>
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</tbody>
</table>

**NGS**

- **ND**
- **RNA virus**

**difficult to detect latent virus?**

*sRNA is an alternative RNA pool for detection of virus.*
Total RNA of Cell bank as Starting Material for Virus Detection by NGS Method

Advantages:
• Both DNA virus and RNA virus can be detected.
• Diversity of host RNA (background including endogenous retroviral RNA) is always stable (ex. Human transcripts <~ 25,000).

Disadvantages:
• Detection of latently infected viruses may be difficult.
• The presence of virus nucleic acid does not always mean the presence of infectious viruses.
• Compared to the PCR method, it takes longer time.
Deep sequencing (NGS) currently requires longer time for virus detection

**PCR**

- 2 h
- 3 h
- total ~5 h

**NGS**

- 2 h
- 2 d
- 3 d
- 1 w
- total ≥2 w

Technical improvements will save time

Development of automatic data processing will save time
Detection of virus nucleic acids in a cell bank by deep sequence (NGS) method

RNA \rightarrow \text{NGS sample} \rightarrow \text{sequencer} \rightarrow \text{HDD}

\text{RNA-seq data} \sim 20-25 \text{ M}

\text{viral-\text{y.3.2}} \ (>16 \times 10^4 \text{ virus seq data})

\text{detected virus seqs}
Cost for sequencing reagent ¥ of virus detection using NGS (HiSeq 2500, Illumina)

<table>
<thead>
<tr>
<th>Reagents / analysis</th>
<th>価格</th>
<th>1 sample</th>
</tr>
</thead>
<tbody>
<tr>
<td>RNA purification</td>
<td>44,500 yen</td>
<td>890 yen</td>
</tr>
<tr>
<td>(RNeasy plus universal mini kit)</td>
<td>44,500 yen</td>
<td>890 yen</td>
</tr>
<tr>
<td>Template preparation kit T</td>
<td>628,000 yen</td>
<td>1,3083 yen</td>
</tr>
<tr>
<td>(TruSeq RNA Sample Prep Kit v2)</td>
<td>628,000 yen</td>
<td>1,3083 yen</td>
</tr>
<tr>
<td>Flow cell</td>
<td>666,000 yen/run</td>
<td>9,250 yen</td>
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<tr>
<td>(TruSeq SR Cluster Kit v3)</td>
<td>666,000 yen</td>
<td>9,250 yen</td>
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<tr>
<td>Reaction reagent</td>
<td>391,000 yen/run</td>
<td>5,430 yen</td>
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<tr>
<td>(TruSeq SBS Kit v3 – HS, 50 Cycle)</td>
<td>391,000 yen</td>
<td>5,430 yen</td>
</tr>
<tr>
<td>RNA-seq virus detection data analysis</td>
<td>- yen</td>
<td>- yen</td>
</tr>
<tr>
<td>total</td>
<td>(28,653 + α) yen</td>
<td></td>
</tr>
</tbody>
</table>

Cost for sequencing reagent: $263/ sequencing
Workflow for detection of adventitious virus sequences

1. RNA-seq data
2. Excluding reads from host transcripts (hg38, etc.)
3. Detect virus-like reads (viral-y3.2)
4. Excluding simple repeat sequences (sr-bg_1.0)
5. Remove cell-specific background (cs-bg_3.0)
6. Identify viruses by mapping etc.
Detection of virus sequences in RNA-seq data
(model cell: HEK293, model virus: FCV, 24 h p.i. at m.o.i. 1)

Yuan & Yusa, unpublished data
Mapping of FCV reads hit by virus database viral-y.2.1

Yuan et al., *unpublished data*
Detection of virus sequences in RNA-seq data
(model cell: HEK293, model virus: FCV, 24 h p.i. at m.o.i. 1)

Yuan et al., unpublished data
Cell specific background reads are obstacles to identify true virus reads

- Uninfected cells have their specific background reads (endogenous virus reads, etc.).
- The background delay final decision of true virus contamination in RNA-seq data.

ex. Background reads in virus-free HEK293 cells (0.36%, 73,356 reads):

- Adenovirus (93.32%): HEK293 cells inherently harbor partial AdV DNA
- HERV-K (1.77%): all of HERV transcripts could not removed by hg38
- Others (4.8%): simple repeated reads, common reads shared between virus and mammalian, etc.
1. HEK293 cell line was established by transfection with Adenovirus type 5 DNA from human fetal kidney (Graham FL et al. J. Gen. Virol. 36: 59–74, 1977).


3. The detected reads were derived from the transcripts of the inserted viral DNA.

Detected Adenovirus Reads Were Mapped in 4.5 kb of Viral Genome

Yuan & Yusa, unpublished data
BVDV Recombination with Bovine Transcript (1)

BVDV CP Rit contains the sequence of bovine Ub and ribosome 40S component protein S27a as a result of recombination with ubi-S27a mRNA derived from the host.

Detection of virus sequences by NGS

Total RNA prepared from a cell bank
rRNA removal
↓
NGS analysis
RNA-seq data
↓
Virus read extraction with virus data base viral-y3.2

Cell-specific background

Infectious virus

background

Infectious virus detection

virus identification by mapping to the viral genome
Efficient Concentration of Adventitious Virus Reads by Subtraction of Host Specific Background (1)

cell: HEK293, virus: FCV at 12 h and 24 h p.i. at m.o.i. 1
Conclusion

The model virus could be detected in RNA-seq data from the infected cells. Subtraction of cell specific database efficiently reduce pseudo positive reads.

Our virus detection pipeline using NGS provide a versatile platform for control of viral safety for biologics including biopharmaceuticals, gene therapeutic products and cell-based products.
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