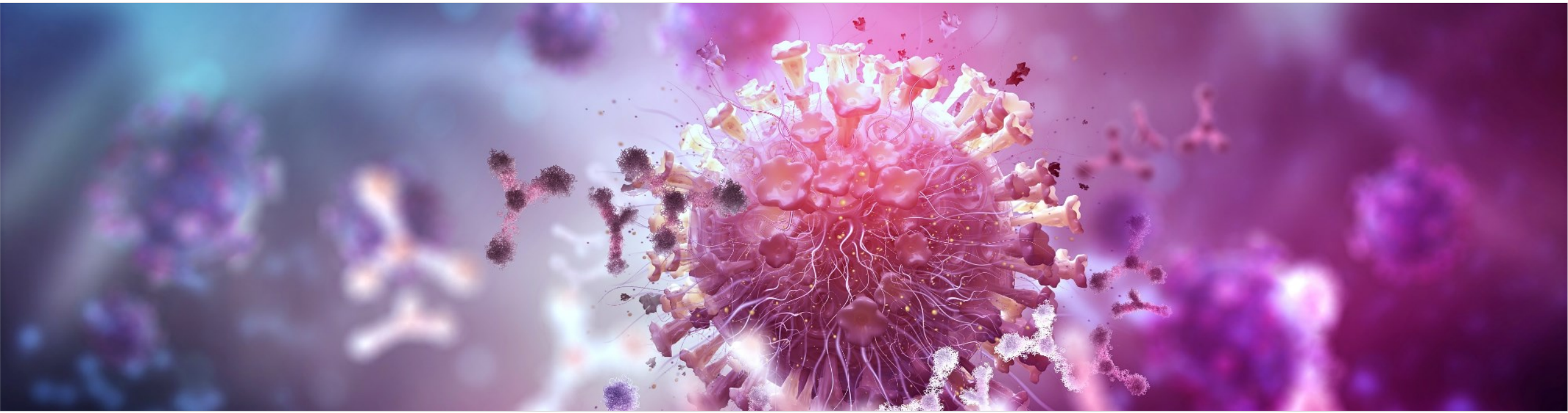
AsahiKASEI BIOPROCESS



Identification of an EHDV Contamination Event in a Biopharmaceutical Product Using a Combination of Adventitious Agent Testing and Next Generation Sequencing

2nd ViruSure Workshop, Vienna, September 2022

Tiffany Avory and Hans-Peter Fasching, ViruSure GmbH

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Presentation Overview

- Introduction to the in vitro adventitious agent test (AAT)
- Identification of a positive AAT test result
  - OOS investigations
  - Additional investigations
- Using Next Generation Sequencing (NGS) as an investigational tool
- Identification of an EHDV contaminant as cause of the OOS
- Conclusions

The History of Virus Contamination Events in Mammalian Cell Culture Derived Biopharmaceuticals

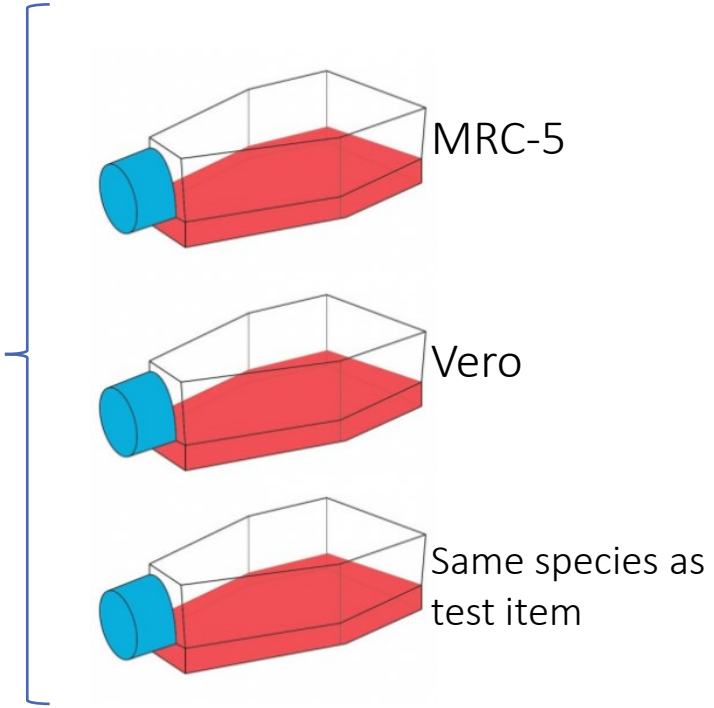
Year of Contamination	Contaminations (virus / host cell)	Total
1985-1989	Bluetongue / CHO EHDV / CHO	2
1990-1994	Herpesvirus / Primary Monkey Herpesvirus / Vero MMV / CHO (x2) Parainfluenza virus / MRC-5 Reo3 / MRC-5 Simian adenovirus / Primary monkey	7
1995-1999	Cache valley virus / CHO Reovirus / Human primary kidney Vesivirus 2117 / CHO	3
2000-2004	CVV / Unknown (x2) Human adenovirus / HEK293	3
2005-2010	CVV / CHO MMV / CHO (x2) Vesivirus 2117 / CHO (x3)	6
2010-Present	MMV / CHO MMV / BHK-21 PCV-1 / Vero	3
Unknown	MMV / BHK-21 Reovirus / Unknown	2
	Total:	26

Data from Barone et. al.; Nature Biotechnology (2020); Vol 38; pp 563-572

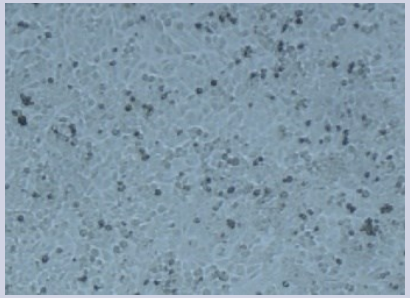
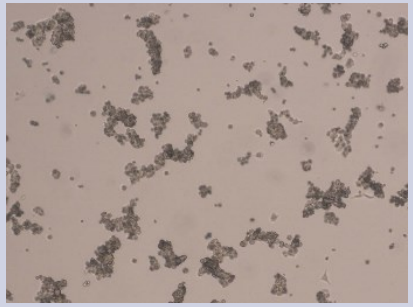
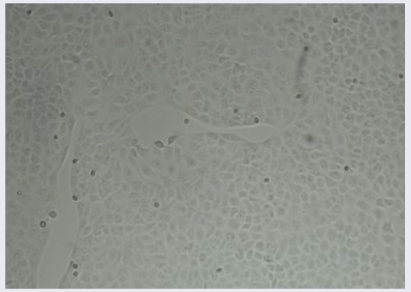
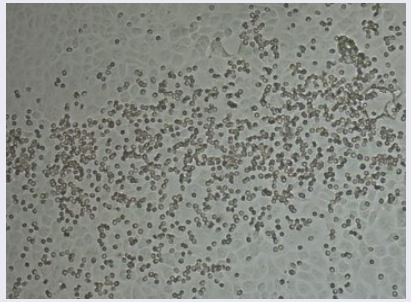
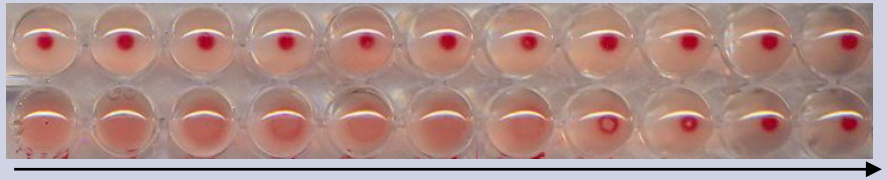
The *in vitro* Adventitious Agent Test

- The *in vitro* Adventitious Agent Test (AAT) is one of the key test for detecting contaminating virus in a sample
- It utilises a minimum of 3 different cell lines to maximise the chance of identifying a virus that would grow on at least one of the cell lines. 3 different end-points are used:
  - Cytopathic effect
  - Haemadsorption
  - Haemagglutination
- The assay has been shown to be significantly more broad ranging than e.g. the *in vivo* adventitious agent test

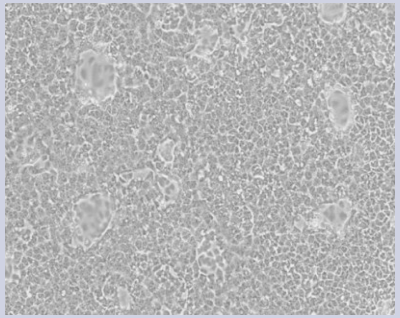
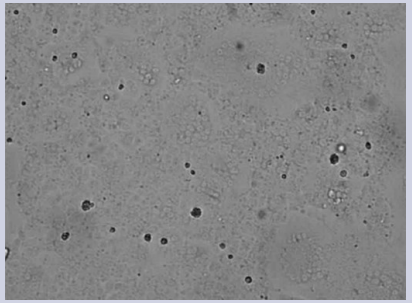
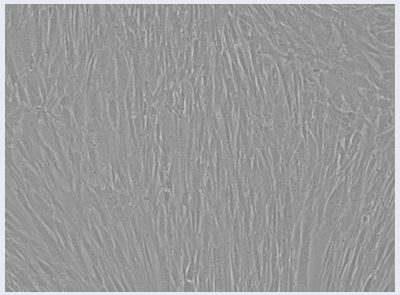
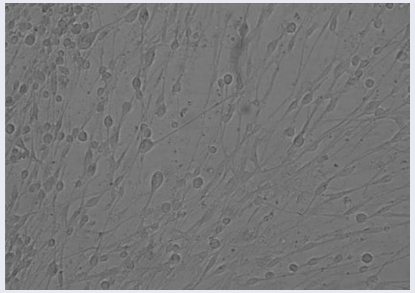
In vitro AAT Assay



In vitro AAT- End points

End point	Examples
Cytopathic effect (cpe)	 Neg  Pos
Haemadsorption	 Neg  Pos
Haemagglutination	Neg  Pos Diln.

Examples of CPE

End point	Examples
Syncitia formation	 Neg  Pos
Cell rounding	 Neg  Pos
Impaired cell growth or productivity	e.g. Vesivirus 2117 -

Cytotoxicity and Interference from Test Matrix

- **Test Sample Matrices may cause an effect on the indicator cell line**
  - Cytotoxic effect – change of morphology, impaired growth or cell death in TS matrix (mild cytotoxic effects may be confirmed by blind passage onto fresh cells)
  - Interfering effect – TS matrix interfers with virus attachment to indicator cells
    – TS is a live virus which replicates in the indicator cells
- **Effects may be overcome by:**
  - Dilution of Test Sample (e.g. 1:10, 1:100)
  - Treatment of Test Sample e.g. Antibody neutralisation
  - Treatment of Test Sample e.g. ultracentrifugation
- **Appropriate Controls important for correct interpretation of the assay**
  - Negative Control - cells inoculated with medium
  - Positive Control – cells inoculated with medium and spiked with positive control virus
  - Interference controls – cells inoculated with Test Sample and spiked with positive control viruses
  - Additional controls for any step or procedure implemented to overcome critical interference

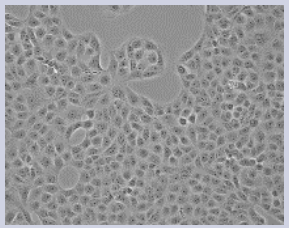
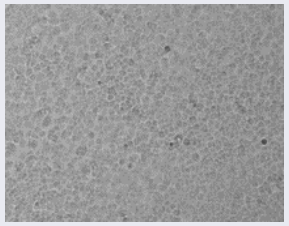
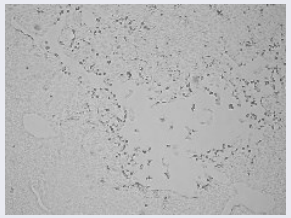
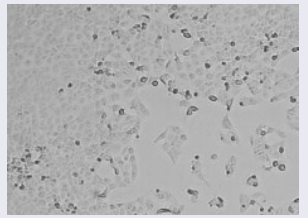
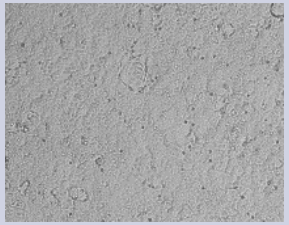
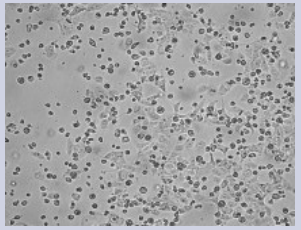
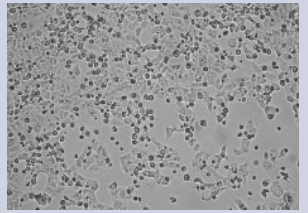
Test & Sample Details

- Bulk harvest sample taken from a bioreactor culturing cells of hamster origin expressing a recombinant protein
- Type of sample: Filtered cell supernatant, negative in sterility and mycoplasma test
- Cells used for in vitro AAT:
  - Vero
  - MRC-5
  - Third cell line was not initiated because of because of OOS in Vero cells
- Test sample was inoculated undiluted, 1:10 and 1:100 diluted (previous known issues with these test samples)

Test Summary (Vero cells)

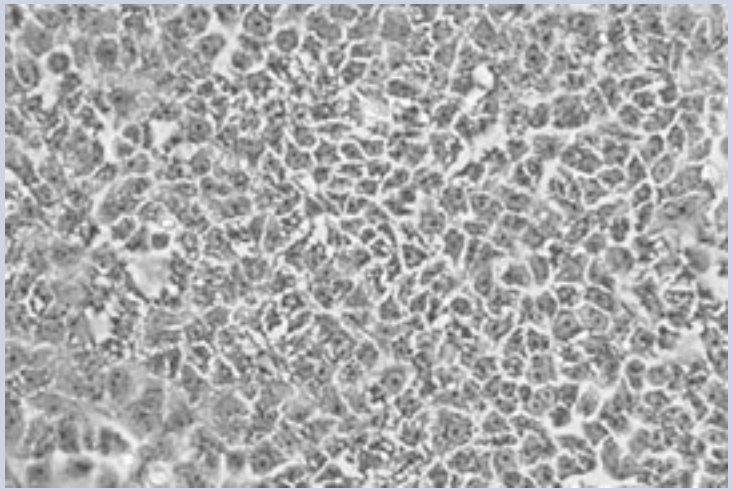
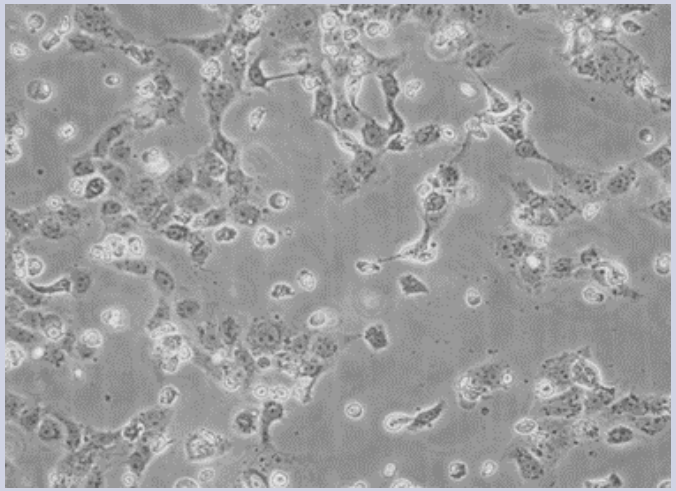
- Immediately after inoculation undiluted sample showed some cytotoxicity but recovered by day 1 post inoculation (1:10 and 1:100 showed no cytotoxicity)
- On day 3 post inoculation in both undiluted and 1:10 inoculated flasks distinct foci could be seen (small holes in cell layer surrounded by dark granular cells). 1:100 was healthy.
- On day 6 post inoculation:
  - Undiluted showed ~80% cpe with very little cell monolayer remaining (flask frozen down for further investigation)
  - 1:10 diluted showed ~50% cpe (flask also frozen down)
  - 1:100 diluted showed many small foci of dark granular cells- flask was sub-cultured at day 7 to observe if cpe could be further cultivated (all cells died quickly confirming cytopathic virus)

AAT Results (cpe)

Day	Negative	Test Sample Undiluted	Test Sample 1:10 diluted
Day 1		As negative control	As negative control
Day 3			
Day 6			

Ruling out matrix effects

- **Highly unlikely due to foci like nature of CPE but must be ruled out as part of OOS investigation**
  - TCID50 titration of harvested 1:10 TS inoculated flask- clear titration pattern and titre of approx. 4.5 log TCID50/ml
  - Blind passage of harvested 1:10 TS inoculated flask onto fresh Vero - clear amplification of effect with >90% CPE by day 4 post inoculation

Day	Negative	Test Sample
Day 4 post-inoculation		

OOS Procedures

Task	Evaluation
Evaluated positive, negative and interference controls?	✓
Evaluate equipment?	✓
Evaluate potential cross contamination from positive controls?	✓
Evaluate possible operater error?	✓
Evaluate correct handling and preparation of the Test Sample?	✓
Evaluate documentation and performance of the assay per SOP?	✓
Evaluate suitability of reagents and materials used for the assay?	✓
Evaluate other extenuating factors (e.g. clear dilution effect with TS)	✓

OOS Investigation Paths?

Method	Pros/Cons
Cell culture?	Long and drawn out investigations with limited specificity
PCR?	Hi specificity but the list of possible PCRs is very long
TEM?	Low sensitivity method but could reveal virus structure
Antibodies?	High specificity but the list of antibodies for neutralisation or fluorescence is very long
NGS?	Requires no knowledge of which contaminant is present, relatively quick investigation but high cost

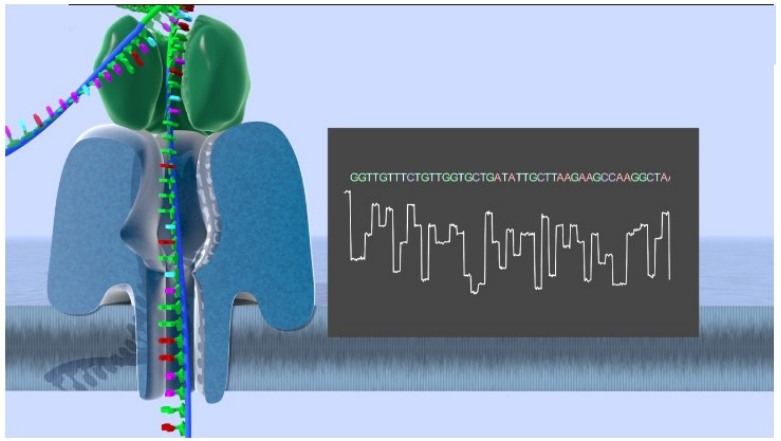
Manufacturers of biopharmaceutical products should have internal procedures defining these paths for the eventuality that a contaminant is identified

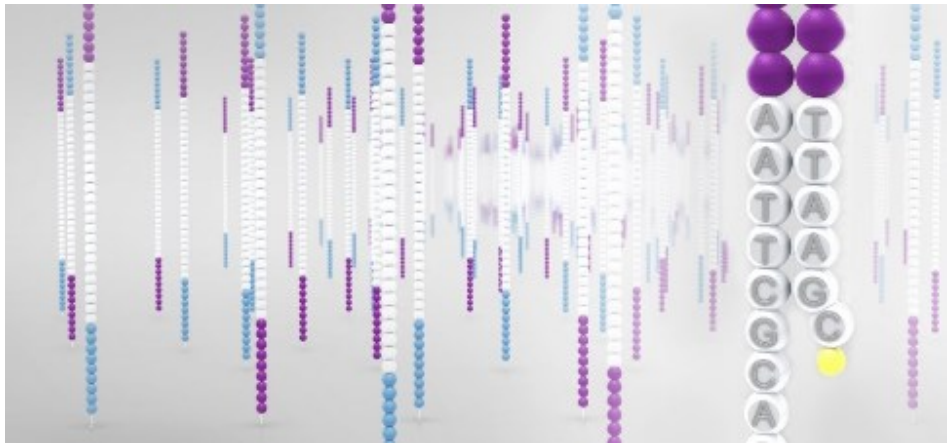
NGS Investigation of AAT OOS

- After identifying the OOS caused by a presumed contaminated sample, we proceeded to further investigate the cause of the positive AAT result using NGS
- As sample for investigation by NGS we chose to use the blind passage harvest:
  - High levels of contaminating agent
  - In the original Test Sample, the levels of the contaminant might have been too low to result in a clear identification
- DNA and RNA extractions of the harvest from the blind passage and negative control cells were prepared for this analysis
- NGS was performed using MinION device from Oxford Nanopore

Oxford Nanopore Sequencing

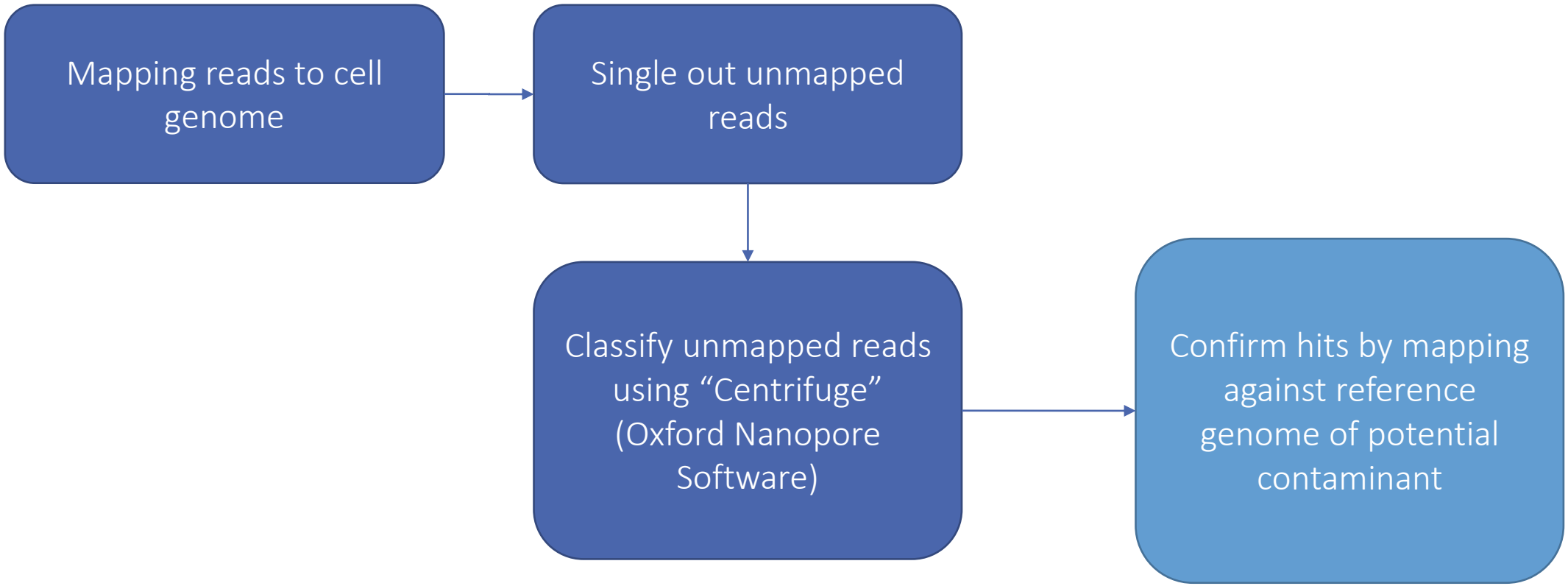
- Oxford Nanopore machines differ from machines like Illumina by running the extracted sample through a pore instead of the “sequencing by synthesis” approach



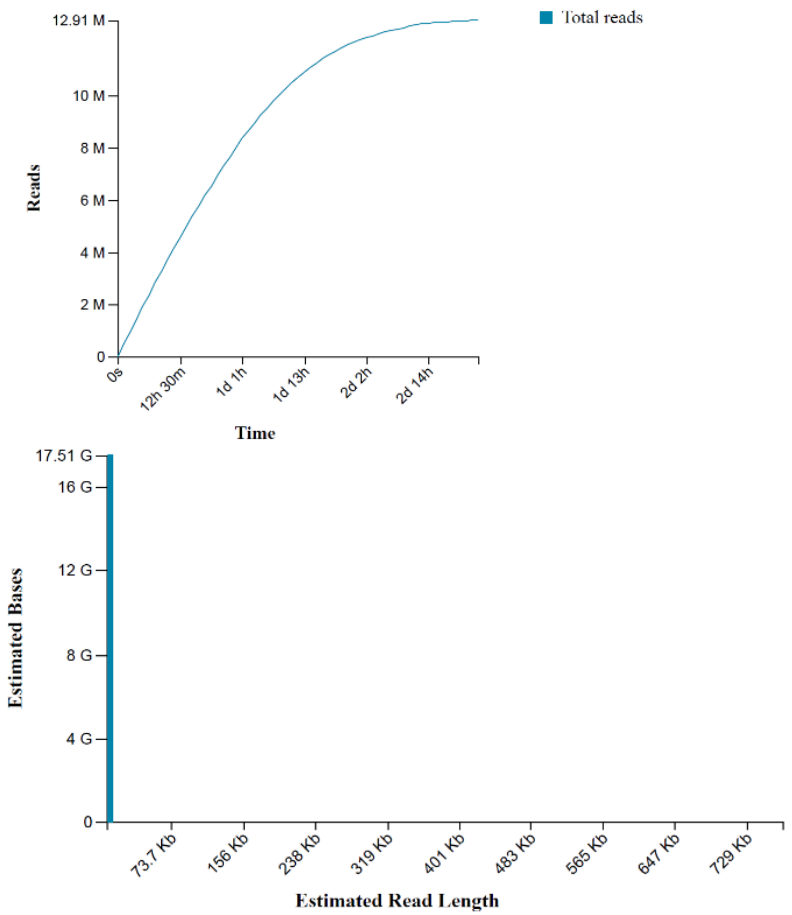


* Images from Illumina and Oxford Nanopore Technologies

Workflow / Data Processing



DNA Analysis



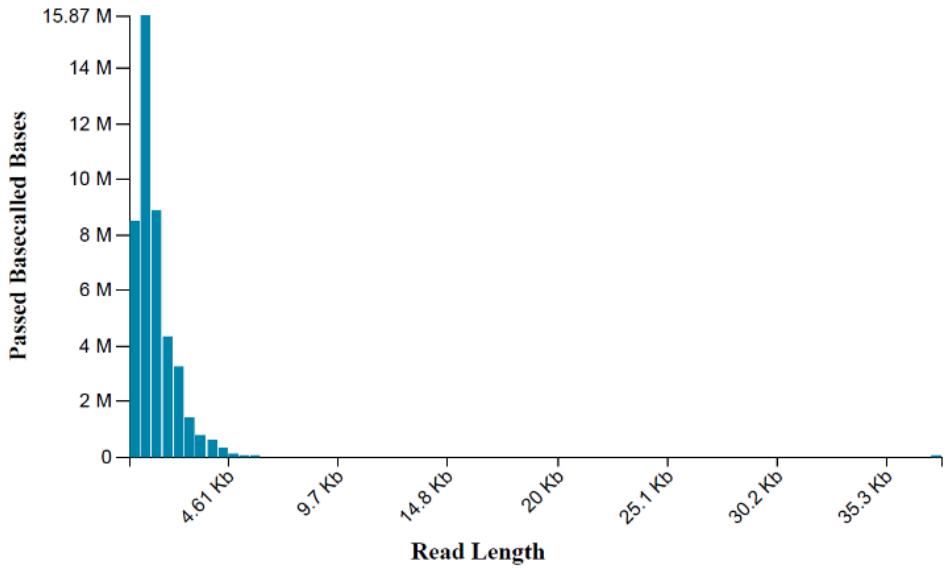
Parameter	Results
Library Preparation	With PCR amplification
Reads Generated	12.9 Mill.
Estimated bases	17.56 GB
Run time	72 hours
Sequences detected	Only retroviral hits were obtained. As they were present in both the Test Sample and the Negative Control, these result from endogenous retroviral signals derived from the Vero cells

RNA Analysis- Optimisation of RT step

- Initial RNA analysis resulted in a low number of total reads but in all runs a low number of reads for EHDV were detected only in the extracted Test Sample
- Possible reasons for the low number of hits were evaluated:
  - Poor recovery during extraction? → Qubit measurement showed acceptable RNA concentrations
  - Poor performance of the Oxford Nanopore MinION flow cell? → QC check performed before each run
  - Poor efficiency of the reverse transcriptase (RT) step?
- RT is an enzyme with high efficiency for the reverse transcription of single stranded RNA. The efficiency of dsRNA transcription is lower. We therefore investigated if denaturation of the RNA prior to the RT step would improve the number of reads (Run #4)

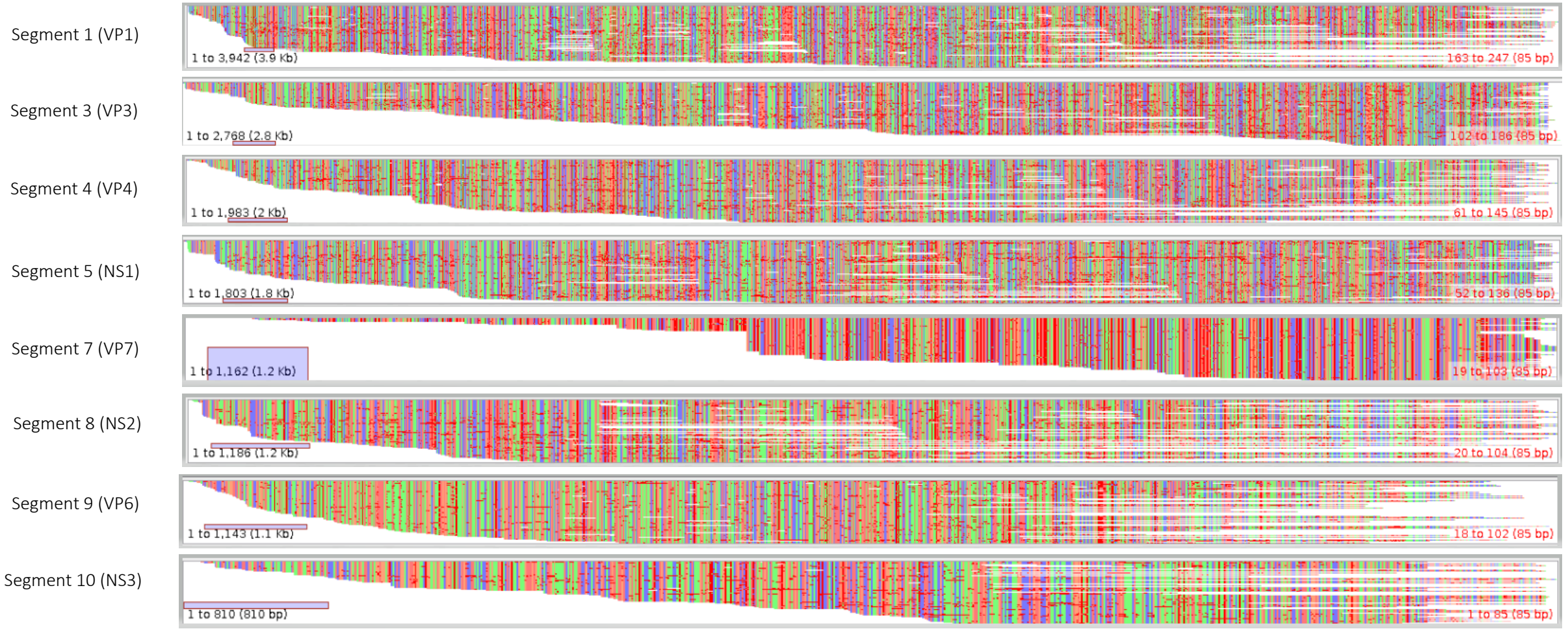
Run #	# EHDV Reads
1st run	3 reads
2nd run	1 read
3rd run	16 reads
4th run	5,300 reads

RNA Analysis (Run #4)

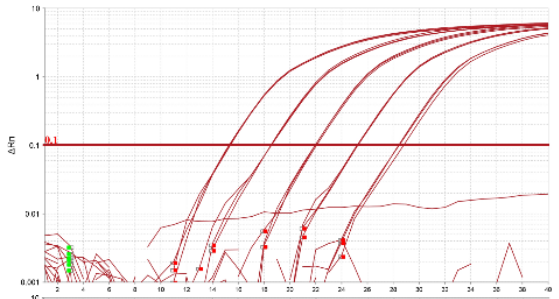
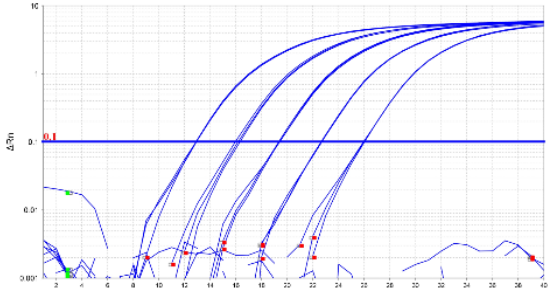
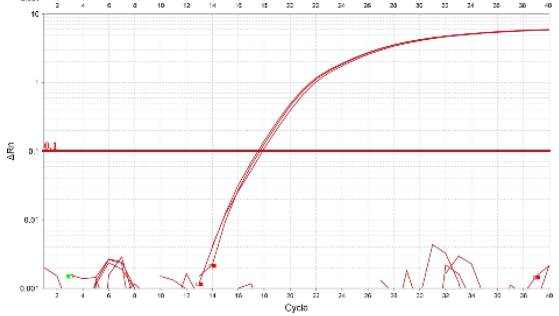
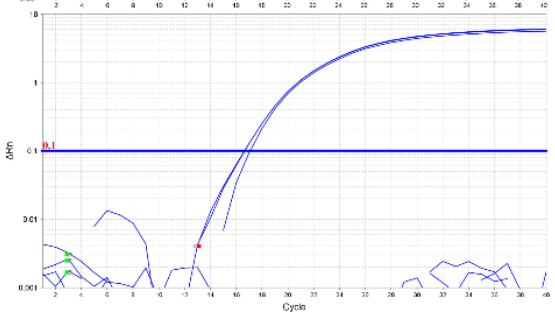


Parameter	Results
Library Preparation	No PCR amplification
Reads Generated	65k
Estimated bases	~55 MB
Run time	23 hours
Sequences detected	Of the ~65,000 reads, 5,300 could be mapped to EHDV sequences (~8% of the reads)

EHDV NGS Data– Alignment to Segments



PCR Confirmation of EHDV

	VP1 Primers	VP6 Primers
Standard Curves		
Test Sample (Harvest from Vero cells inoculated with 1:10 diluted sample)		

	VP1 PCR Titre	VP6 PCR Titre
PCR Results:	~8.06 $\log_{10}$ gc/ml	~7.56 $\log_{10}$ gc/ml

EHDV/Reoviruses

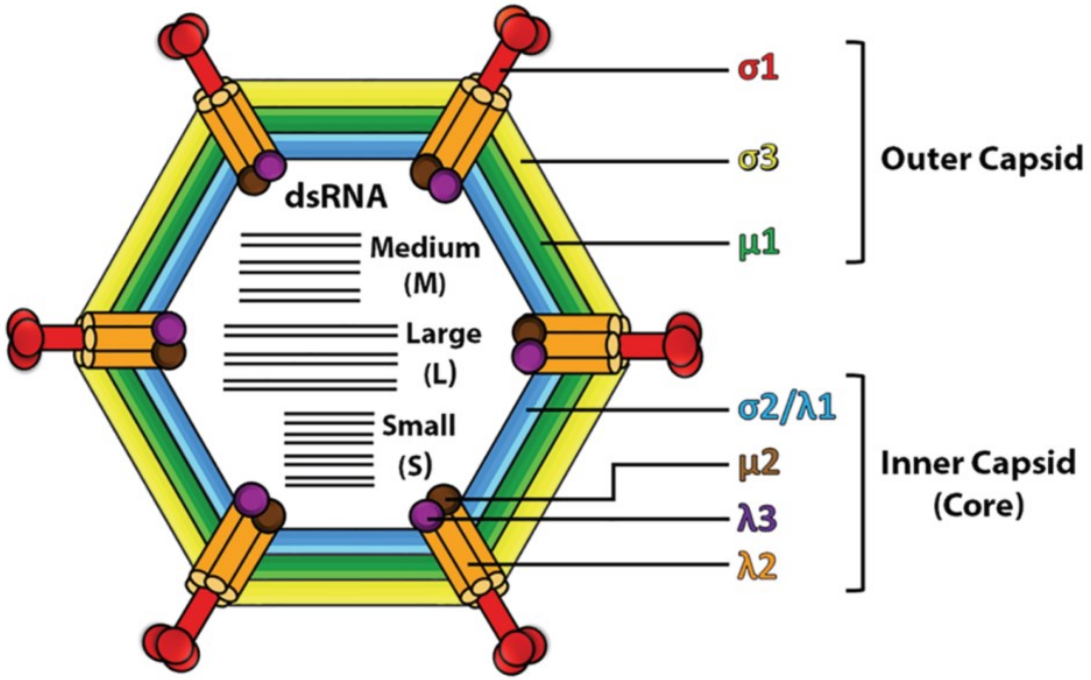
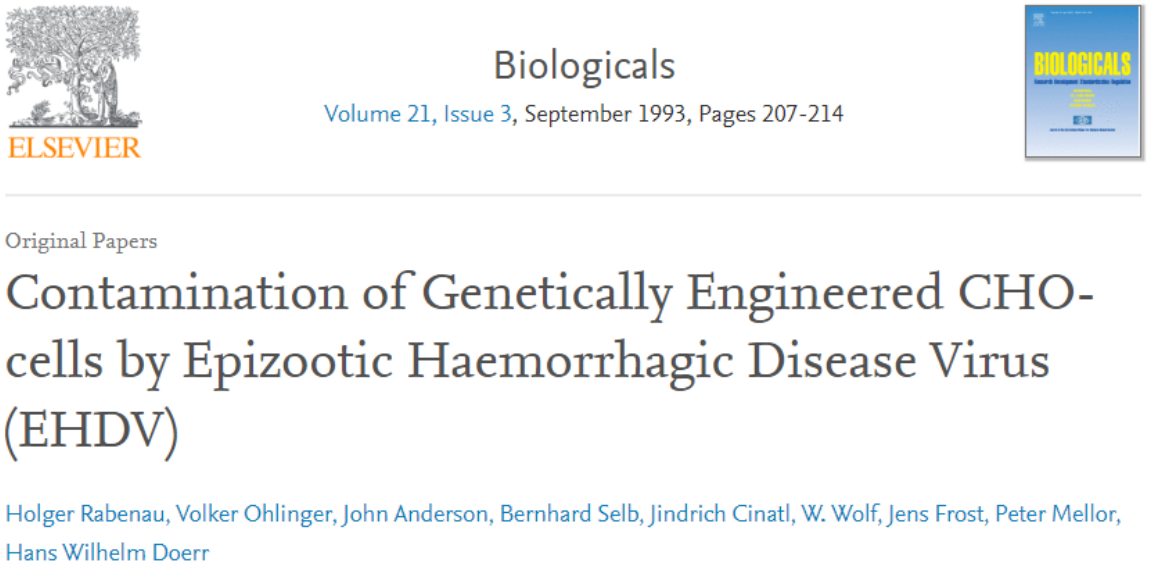


Image by Adil Mohamed, Randal N. Johnston, and Maya Shmulevitz *

- EHDV is a virus that is endemic in most parts of the world including Europe, Americas, Asia and Australia
- The virus is absent from New Zealand (ecologically isolated islands)
- Belongs to the family *Reoviridae*: non-enveloped double stranded segmented RNA viruses
- EHDV has been detected previously as a contaminant of recombinant cell cultures

* https://www.mdpi.com/1999-4915/7/12/2936/htm

EHDV Contamination of Biologics?



ELSEVIER

Biologicals

Volume 21, Issue 3, September 1993, Pages 207-214

Original Papers

Contamination of Genetically Engineered CHO-cells by Epizootic Haemorrhagic Disease Virus (EHDV)

Holger Rabenau, Volker Ohlinger, John Anderson, Bernhard Selb, Jindrich Cinatl, W. Wolf, Jens Frost, Peter Mellor, Hans Wilhelm Doerr

- EHDV and other Reoviruses like e.g. Bluetongue will be a risk concern where bovine serum/components are present in the culture medium
- Reoviruses have a high titre viraemic phase so even one infected cow will result in significant levels of contamination in pooled serum
- The risk can be significantly reduced through testing followed by inactivation (e.g. gamma irradiation)

Summary

- We have identified a new case of EHDV contamination in a bulk harvest from a recombinant fermenter
  - High number of reads following protocol optimisation
  - Confirmed using EHDV specific qPCR
- NGS proved to be an effective tool for identifying the contaminant responsible for CPE in the AAT test
  - Analysis was possible with even a low-throughput MinION flow cell

Thank you for your attention

Questions?