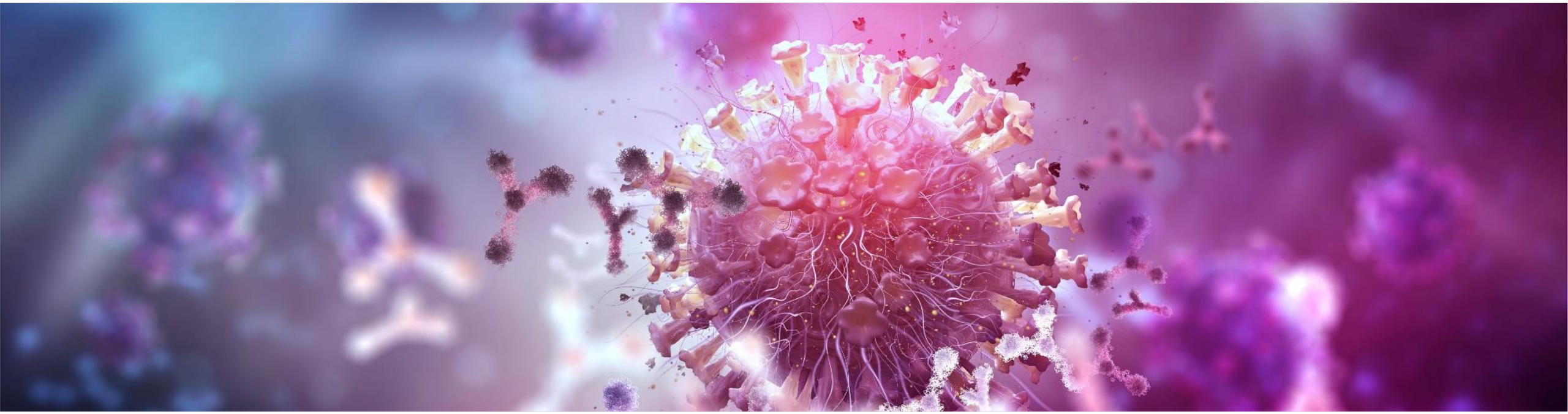

Case Studies for Rapid Identification of Adventitious Agents and the Importance of Raw Material Sourcing

ViruSure Workshop; April 8th 2024

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Presentation Overview

1. Controlling sourcing and upstream risks
2. Testing: Techniques for Adventitious Agent Detection
3. Case Study 1
4. Case study 2
5. Lessons learned from previous contaminations
6. Summary

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

At ViruSure we have identified two additional culture based contamination events in the last 2 years which will be presented in later slides

What were the Sources of Contamination in these Historical Events?

Contaminated Cell Line	Contaminating Virus	Pathogenic to Humans?	Suspected and Confirmed Sources of Contamination					
			Serum	Recombinant Medium Component	Undetermined Medium Component	Operator	Host Cell Line	Not Found
Viruses found to contaminate CHO cell culture								
CHO	Bluetongue virus	No	1					
CHO	Cache valley virus	Yes	2					
CHO	Minute virus of mice	No		1	3			1
CHO	Vesivirus 2117	No	4					
Viruses found to contaminate human or primate cell lines								
Primary monkey, Vero	Herpesvirus	Yes				1	1	
HEK293	Human adenovirus type 1	Yes				1		
MRC5	Parainfluenza virus type 3	Yes				1		
MRC5	Reovirus type 3	Yes				1		
Primary monkey	Simian adenovirus	No					1	

Comments:

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

Upstream Virus Contamination Risks

- Recombinant Products-Steps are implemented downstream of cell culture to reduce virus risk and medium is often chemically defined:
  - Chemically defined though does not necessarily mean there is no virus risk

- Cell or virus based therapies- Steps downstream for controlling virus risk may not be feasible
  - Virus might be inherently carried in the cells (latent or inapparent infections)
  - Medium is often complex including human or animal derived components where the virus risk is higher (FBS or purified bovine/porcine proteins, PDGF)

    → there is a greater need to control the virus risk in such components

- The impact of a contamination event upstream can be significant:
  - Supply of product is impacted
  - Significant investigation / clean-up costs
  - Impact on company image

ICH Q5A (R2)

Sourcing and Upstream Controls:

- *Manufacturers should avoid using human- and animal-derived raw materials (e.g., human serum, bovine serum, porcine trypsin) in their manufacturing processes when possible. When this is not possible, the use of animal-derived raw materials should be supported by the relevant documentation or qualification of the material, commensurate with risk. Information such as the country of origin, tissue of origin, virus inactivation or removal steps applied during the manufacturing process of the material, and the types of virus testing that have been performed on the raw material should be provided.*
- *When possible, cell culture media or media supplement treatments such as gamma irradiation, virus filtration, high temperature short time processing, or ultraviolet C irradiation can be used as additional virus risk mitigation measures.*

- Advanced detection technologies such as NGS and PCR play an important role in the new ICH Q5A document and can be used as replacements for in vivo and in vitro adventitious agent testing ***without any direct head-to-head*** comparison

MMV and Vesivirus 2117 Contaminations from environmental sources

MMV Contamination events started to be noted by Genentech in the 1990's in CHO fermenters

- Multiple contamination events (not just a single isolated event)
- A number of other manufacturers have also had incidences of contamination (maybe as many as 50% of the large manufacturers!)
- Source of contamination was never clearly identified, but it was assumed to be caused by facility rodents: present in GMP facility or suppliers for excipients or media components (i.e. fomite transmission)?

Timing of Vesivirus 2117 contamination events:

- ~1998- Boehringer Ingelheim
- 2008- Genzyme had two contamination events around the same time with different 2117 strains in the US and Belgium:
- No identifiable shared components in use at both facilities
- 2009- Genzyme reported a second contamination with the same strain as found in the 2008 US contamination event
- Direct data to support a bovine origin for the contamination is limited
- Phylogenetic analysis shows closest relationship with Canine Caliciviruses

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

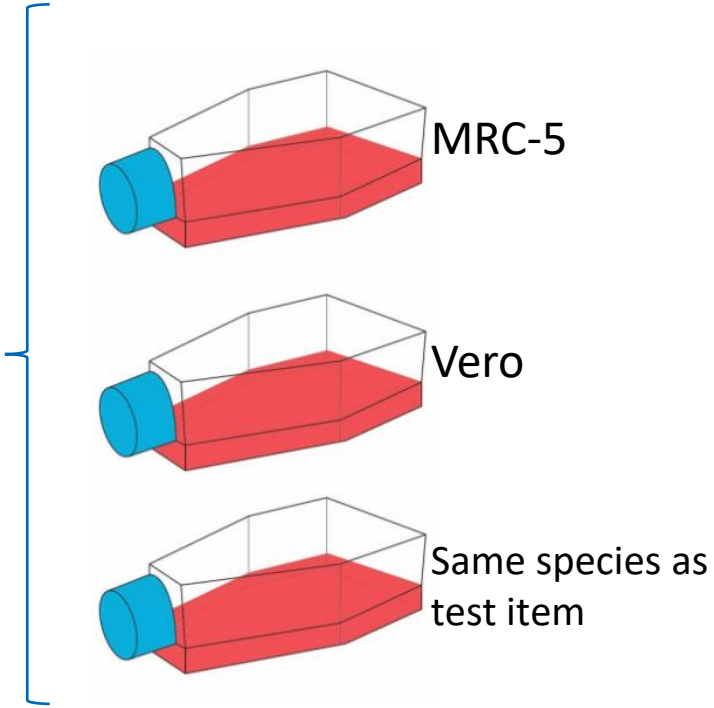
It utilises a minimum of 3 different cell lines to maximise the chance of identifying a virus that would grow on at least one of the cell lines. 3 different end-points are used:

- Cytopathic effect
- Haemadsorption
- Haemagglutination

The assay has been shown to be significantly more broad ranging than e.g. the *in vivo* adventitious agent test

However silent infections demonstrate that the standard *in vitro* adventitious agent assays carry the risk that not all viruses will be detected

In vitro AAT Assay

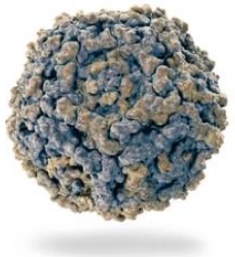


Molecular Techniques for detection of Adventitious Viruses

- PCR

May have comparable or better LOD to the infectivity test- high particle to infectivity ratio for most viruses

Possible to screen for a large panel of viruses- viruses selected for PCR testing should be based on a risk assessment



- NGS

Based on the approach used an indication of infectivity can be determined

  - Genomics, received hits have to be checked on whether they are indeed derived from replicating virus
  - Transcriptomics focuses on RNA only present in the sample. Picking up a signal in a transcriptomics approach already tells that the virus is also replicating
  - Viromics approach focuses only on intact viral particles giving already an indication that a pick-up signal might be from a live virus.

2 case studies of adventitious agent detection at ViruSure

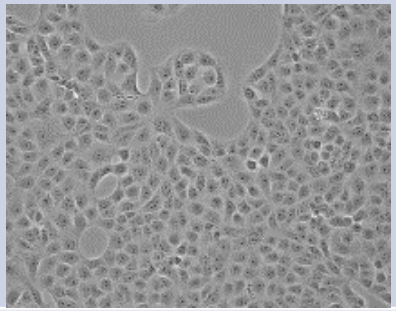
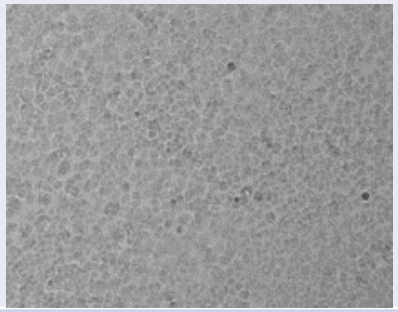
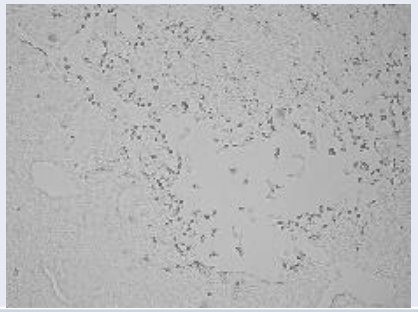
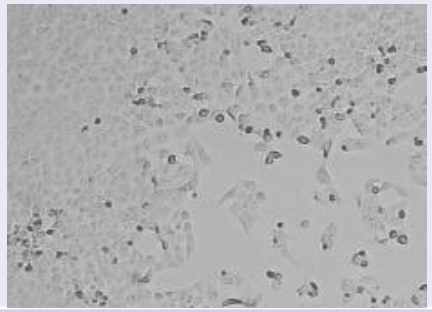
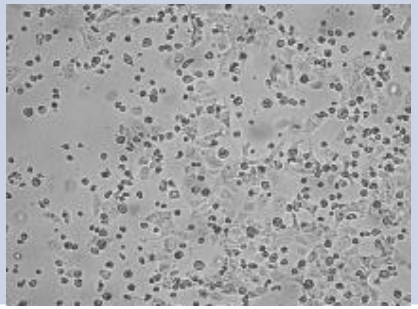
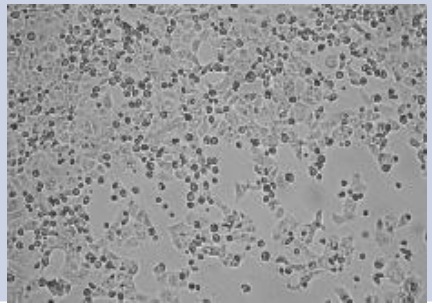
Case Study 1

- 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)

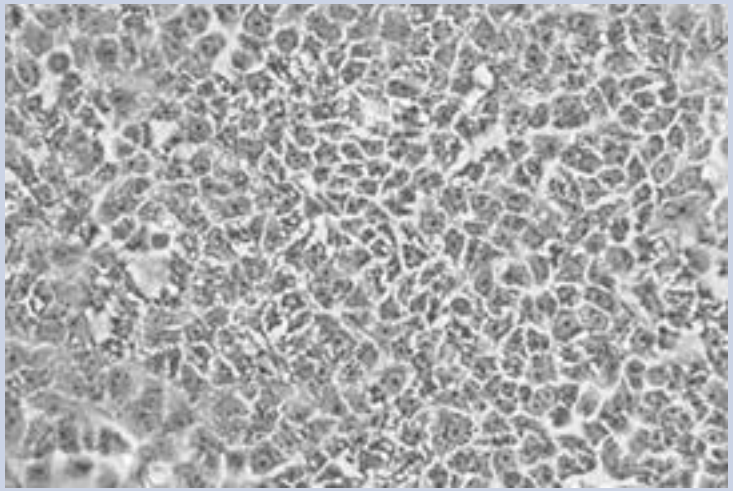
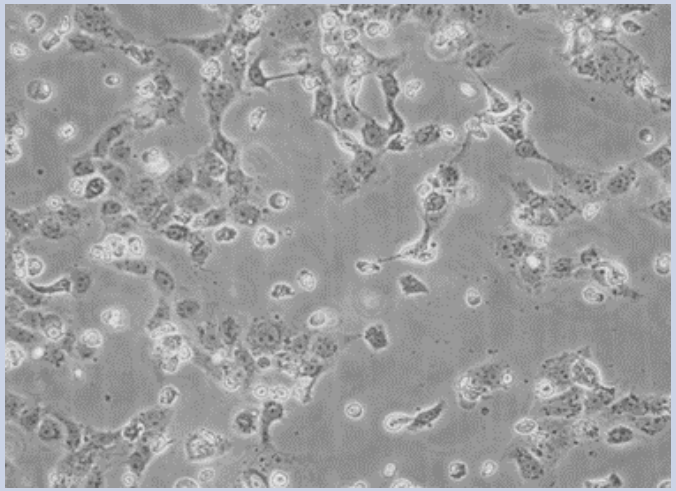
- Immediately after inoculation undiluted sample showed some cytotoxicity on both cell lines but they recovered by day 1 post inoculation (1:10 and 1:100 showed no cytotoxicity)
- On day 3 post inoculation in both undiluted and 1:10 inoculated flasks distinct foci could be seen (small holes in cell layer surrounded by dark granular cells). 1:100 was healthy.
- On day 6 post inoculation:
  - Undiluted showed ~80% cpe with very little cell monolayer remaining (cells and supernatant were frozen down for further investigation)
  - 1:10 diluted showed ~50% cpe (also frozen down for further investigation)
  - 1:100 diluted showed many small foci of dark granular cells

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		

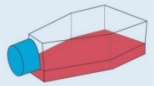
OOS Procedures

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 for identification of contaminant

OOS Investigation

Identification of contamination



Cell Culture

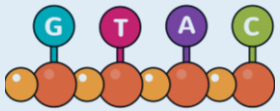
- Limited specificity
- Long

TEM

- Reveals the virus structure
- Low sensitivity

PCR

- High specificity
- Extensive list of potential qPCR



NGS

- High specificity
- Sequence agnostic
- Quick TAT

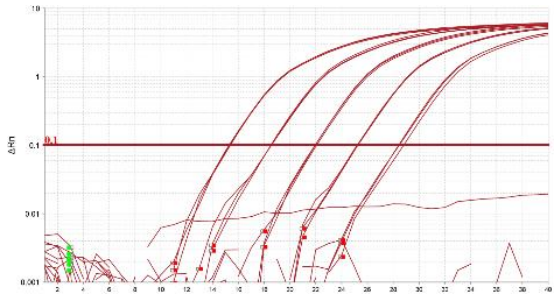
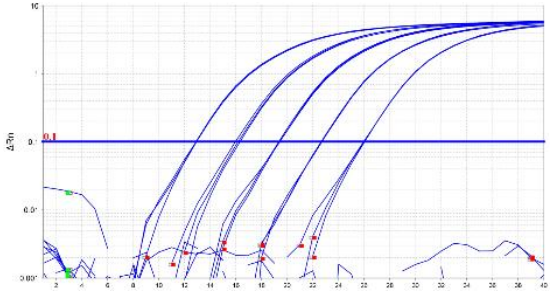
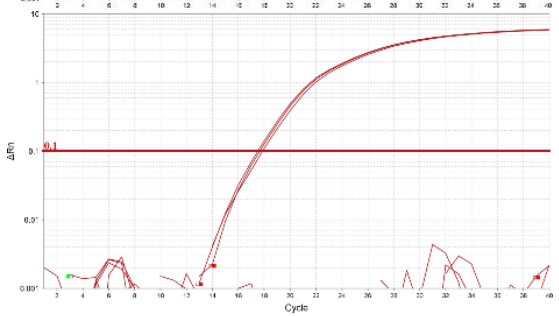
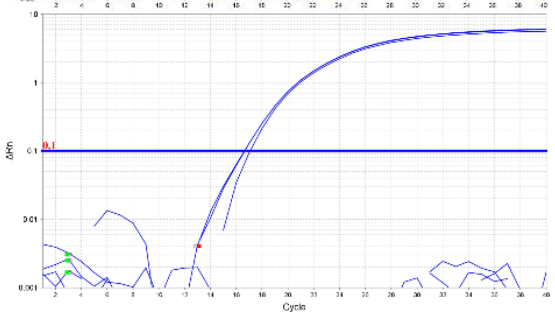
Manufacturers of biopharmaceutical products should have internal procedures defining these paths for the eventuality that a contaminant is identified

NGS testing of AAT sample

- Used sample taken from blind passage of 1:10 inoculated flask that was showing 90% CPE at day 4 as this likely represented the highest titre
- DNA analysis: 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: initial 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 low number of hits was evaluated and poor efficiency of the RT step was identified
- 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

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

- 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
- 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)

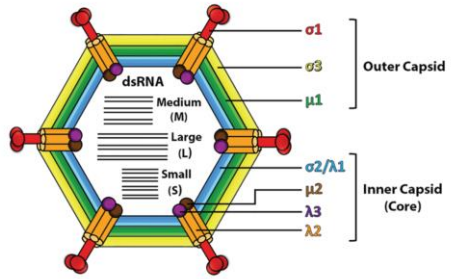
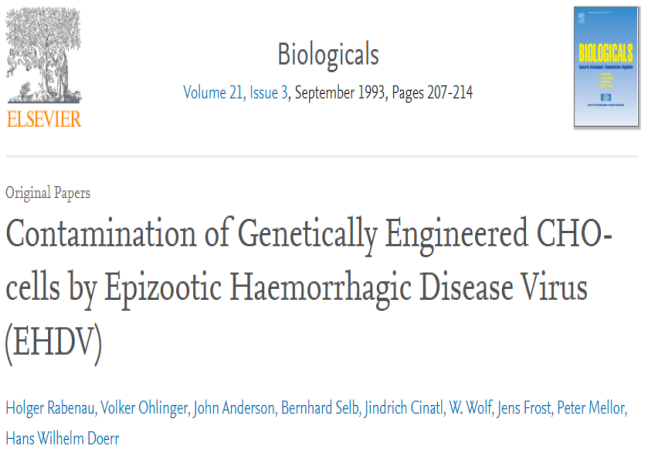


Image by Adil Mohamed, Randal N. Johnston, and Maya Shmulevitz *


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

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

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Case Study 2

- Cell bank Testing
- Type of sample: Filtered cell lysate, negative in sterility and mycoplasma test
- Cells used for in vitro AAT:
  - Vero
  - MRC-5
  - Same species as Test Sample
- Test sample was inoculated undiluted only

AAT Summary (Vero cells) and NGS identification

- On day 3 post inoculation: round granular cells were seen on the surface of the cell layer
- On day 7 post inoculation: round granular cells persisted and flasks were sub cultured
- On day 9 post inoculation: clear difference between negative and Test Sample cells. Test Sample cells less confluent and showing more granulated cells. The cells were harvested for blind passage to rule out cytotoxicity
- Blind passage of day 9 sample showed clear amplification of effect with strong CPE at day 3 post-inoculation
- NGS performed using lessons learned from case study 1
- Contaminant identified – picornavirus - most likely source animal derived component used in production of cell bank, not part of most standard qPCR packages highlighting the advantage of a sequence agnostic approach such as NGS for the future of AAT testing

Lessons learned from past and recent contamination events

- The contaminations detected by ViruSure in the last 2 years show such events are still occurring and highly specific methods such as standard PCR packages may miss such contaminations, a sequence agnostic approach is therefore more suitable
- Previously reported contaminations demonstrate that the use of animal-origin free media and supplements is not a fix all solution as adventitious agents may be introduced from other sources
- Global climate change may affect the spread of arboviruses resulting in new patterns of viral contaminants in animal derived materials
- Handling of MMV and Vesivirus 2117 contamination events by Genentech and Genzyme in an open and transparent way allowed the whole industry to address the issue.
- Where viral agents of concern are identified and shared, the industry can respond quickly and effectively to emerging patterns or previously unseen agents with highly specific test methods such as PCR

Summary

- We have identified 2 new cases of adventitious agent where the most likely root cause is contamination of animal derived raw materials
- NGS proved to be an effective tool for identifying the contaminant responsible for CPE's in the in vitro AAT test
- Combining cell culture detection and NAT may be appropriate in some cases
- Manufacturers of biopharmaceutical products should have internal procedures defining paths for the eventuality that a contaminant is identified regardless of the test method used for identification
- Disclosure of detected viruses would allow emerging patterns to be tracked and facilitate preparation of up to date risk assessments
- Sourcing of raw materials, especially animal derived components is vital to reduce the risk of contamination events e.g. gamma irradiated FBS, heat treated porcine trypsin

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감사합니다 Gracias Danke

Благодаря 谢谢 Tack धन्यवाद Dziękuję

Спасибо Thank You Obrigado

Děkuju Grazie Ευχαριστώ Merci Köszönöm

ありがとうございました Teşekkür ederim

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