Infectivity vs qPCR testing

Advantages, Challenges and Conclusions

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Natascha Hodosi, Head of Molecular Biology, ViruSure

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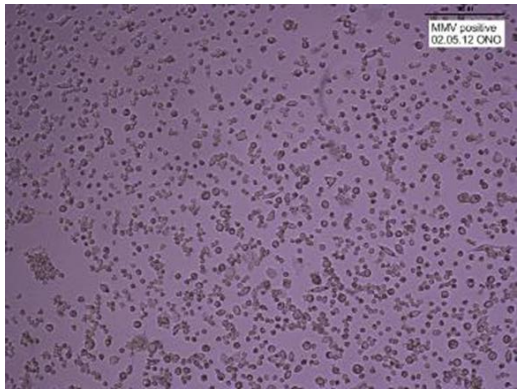
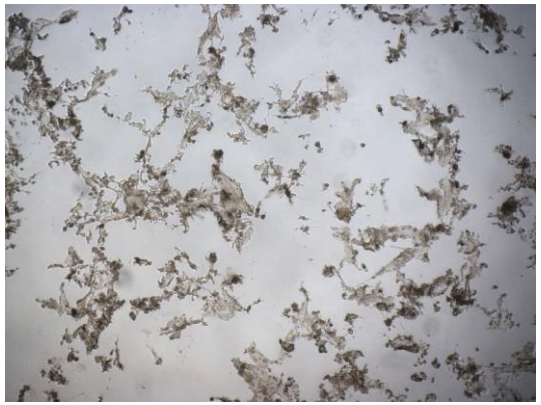
Content of presentation

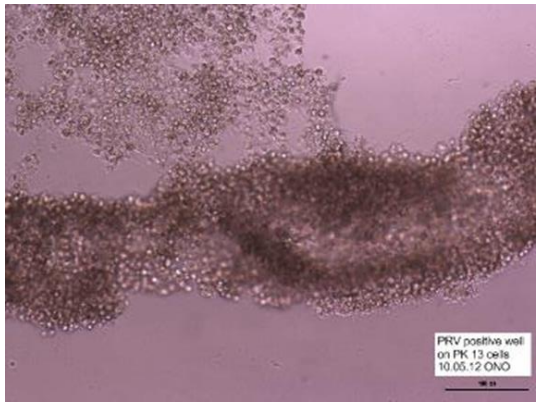
1. Overview of tests (TCID50, AAT, qPCR)
2. Applications
3. Direct comparison Infectivity vs qPCR
4. Conclusion & Summary

TCID50

- Viruses cause morphological change in susceptible inoculated cells
- These changes are known as a cytopathic effect (CPE)
- The appearance of a CPE depends on the virus and the host cell line on which it is grown

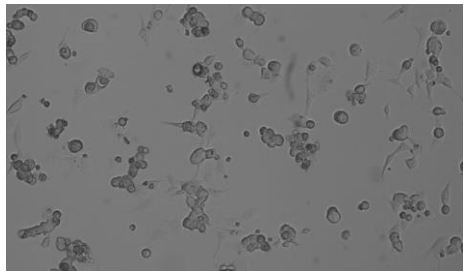






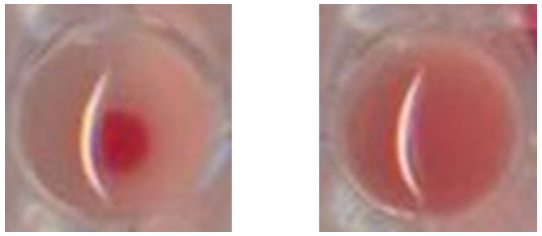
In vitro testing (AAT)

- based on the ability of cell cultures to grow a wide array of pathogens
- Selection of the cell line depends upon the origin of the sample
- different adventitious viruses (cytopathic viruses, haemadsorbing viruses and haemagglutinating viruses)

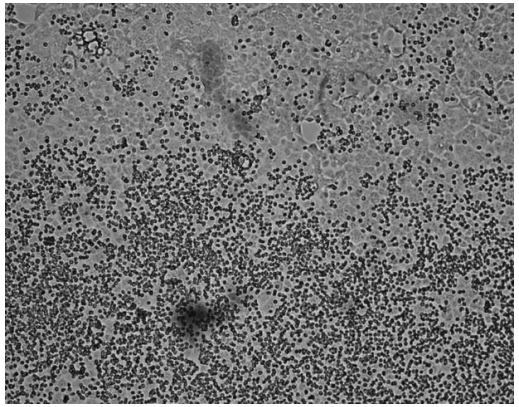


CPE

NEG POS



Hemagglutination (virus binds to the surface of RBCs)



Hemadsorption (Red blood cells will adhere to infected cells)

qPCR

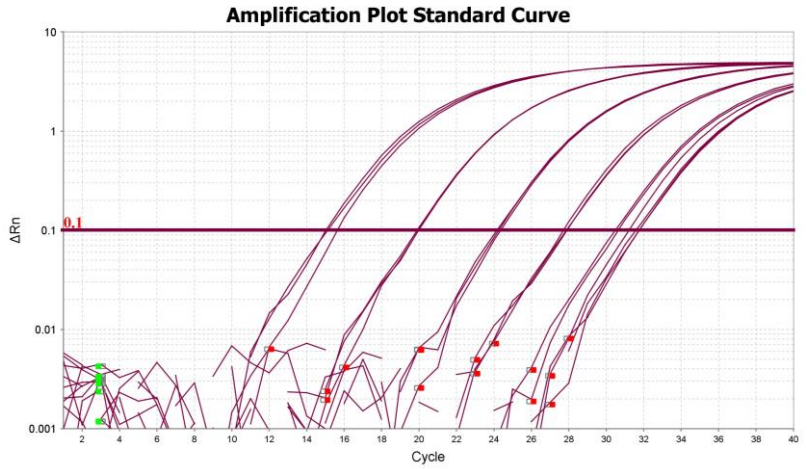
- Fast, selective and sensitive method to amplify low amounts of DNA/RNA

- Consists of three steps (same as PCR), for RNA an RT step is done first:

Denaturation: dsDNA fragments are separated through heating

Annealing: primers can anneal to the template

Extension: a new DNA strand is synthesized



- fluorescent reporter dyes are used:

- intercalating DNA dyes (e.g. Sybr Green) or

- sequence specific labelled probes

- The increase in fluorescent signal is proportional to the amount of synthesized DNA during each cycle

Applications for Products, Raw Material and Clearance Studies

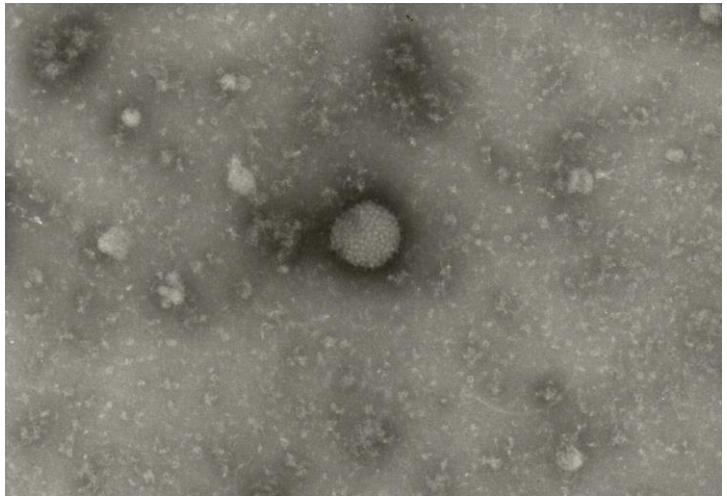
Product Testing Requirements

Product type	Potential risk	Risk control measures
API (manufactured with animal derived products)	Limited virus removal Animal virus risk Incorrect sourcing/testing prior to manufacture	Elimination of animal derived components Testing for animal derived viruses Incorporation of effective virus clearance steps
Cell therapy	Human viruses Endogenous Retrovirus Latent virus infection	Donor selection/Screening Testing for active/latent viruses
Gene therapy vectors	Interference in *in vitro/in vivo* adventitous agent assays Replication competent virus contamination	Neutralisation of virus prior to testing Testing for replication competent viruses
Live virus vaccines	Interference in *in vitro/in vivo* adventitous agent assays No dedicated virus removal steps	Neutralisation of virus prior to testing Use of virus specific qPCR assays

Bulk Harvest Testing with TEM (transmission electron microscopy)

- Regulatory requirements from ICH Q5A to determine the retroviral particles in unprocessed bulk

- **TEM**
  - General industry standard
  - Images of both intact and damaged viral particles
  - external features (eg shapes) and internal structures (eg nucleic acids) can be seen
  - High variability and low sensitivity (~10^5-10^6 particles/ml)
  - Requires concentration or processing of the sample



Bulk Harvest Testing with FPERT (Fluorescent Product-Enhanced Reverse Transcriptase Assay)

- PCR based assay: Detects RT activity associated with particles
- High sensitivity (~10 retrovirus particles) and more reproducible
- Must be validated against the TEM method to be accepted by regulatory authorities
- Useful for cell lines, viral vaccines and gene therapy vectors

Bulk Harvest Testing - Issues

FPERT

- Native cellular DNA polymerases possess RT activity, therefore samples need to be filtered and centrifuged before freezing. Additionally, ultracentrifugation is recommended
- Cell lines (e.g. CHO) can express low levels of an endogenous RT activity which cannot be inhibited by activated calf thymus DNA or an ultracentrifugation step

TEM:

- Extensive sample preparation required (UC, fixation)
- Time consuming
- Virus or virus-like particles are not distributed uniformly which could lead to an overestimation
- Low sensitivity

Bulk Harvest Testing- RVLP (retrovirus-like particles) qPCR

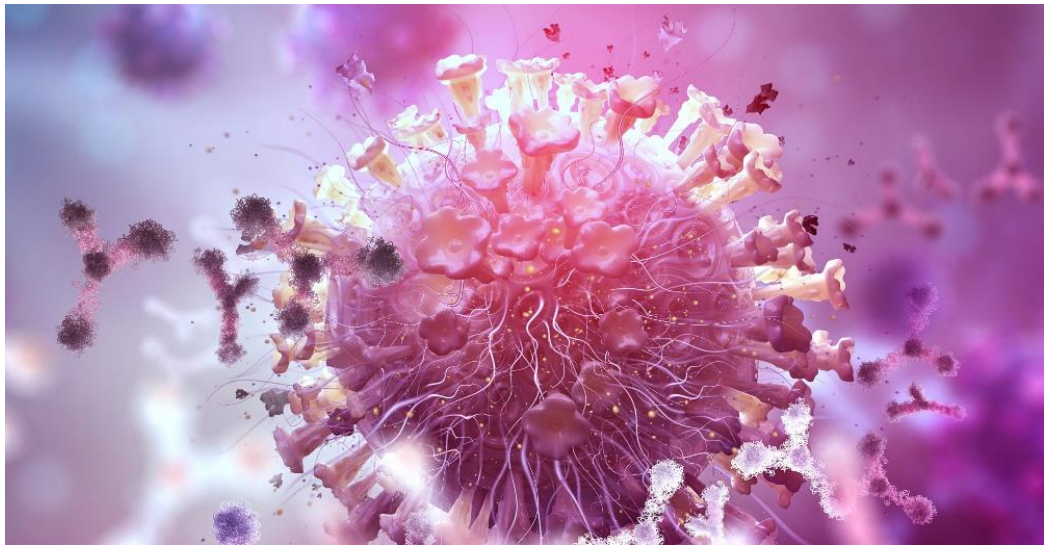
- Possible replacement of other methods
- Quantification of viral RNA
- Higher sensitivity as TEM
- Involves RNase digestion of cell culture fluid to remove free RNA, followed by extraction of total nucleic acid and digestion with DNase to remove extracted DNA molecules, and then finally qPCR including an RT step
- Empty capids and cell debris are excluded from the quantification

Live Vaccines & Virus Vectors

- For live virus vaccines or replication competent gene therapy vectors, issues may be encountered with cell-based detection systems where the virus is able to replicate on the detector cells
- CPE could mask the end point for cell-culture based assays
- Neutralisation of the replication competent virus may be required
- Even with neutralizing serum, some viruses cannot be completely inactivated: Additional dilution of the sample may be necessary to achieve complete neutralization, reducing the sensitivity of the assay
- Need to test by PCR is evaluated on a case-by-case basis

Live Vaccines Example

- Modified vaccinia Ankara (MVA) is an attenuated strain of the vaccinia virus which used against smallpox
- Testing for bovine viruses is required as FBS is used for cell culture (see next slide)
- qPCR testing showed a positive signal for BVDV-1
- Gamma-irradiation is not sufficient to destroy RNA
- 0.1 µm filtration was introduced to remove MVA (~ 300 nm) before using the sample for AAT



Testing of Bovine Serum

- Traditionally FBS has been tested for a number of bovine viruses using an infectivity test with 3 end-points:
  - Cytopathic effect
  - Immunofluorescence
  - Haemmagglutination/Haemadsorption

- PCR assays have gained momentum in recent years:
  - Comparable or better LOD to the infectivity test- high particle to infectivity ratio for most viruses
  - Possible to screen for a larger panel of viruses- viruses selected for PCR testing should be based on a risk assessment
  - Positive qPCR signal in FBS, but not infectious virus if gamma-irradiation was performed

viruses MDPI

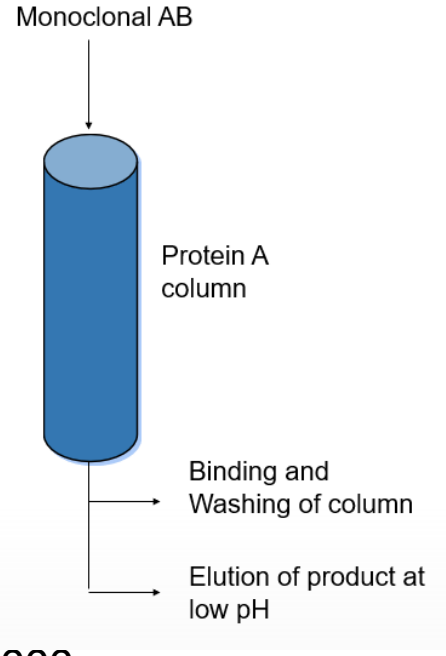
Brief Report

Virome Characterization in Commercial Bovine Serum Batches—A Potentially Needed Testing Strategy for Biological Products

Willian P. Paim [1,2], Mayara F. Maggioli [1], Shollie M. Falkenberg [3], Akhilesh Ramachandran [4], Matheus N. Weber [5], Cláudio W. Canal [2] and Fernando V. Bauermann [1,*]

qPCR Testing in Viral Clearance Studies

- Infectivity assays remain the gold standard
- qPCR data is used as supporting data
- Several points need to be considered



Particle to infectivity ratio

- Most viruses have a high particle to infectivity ratio in the order of 1:100 up to 1: 10 000
- In a viral clearance study, it is desirable to control if the removal of non-infectious particles follows the same pattern as infectious particles

qPCR Testing in Viral Clearance Studies

- Interference controls

Controls must be included both for extraction and qPCR to demonstrate that the sample is not interfering

- free vs. encapsulated DNA/RNA

Any DNA/RNA present in a sample which is not inside a virus capsid should be removed by nuclease treatment

This is needed for inactivation steps, where the nucleic acid might not be destroyed; or for removal steps (eg nanofiltration) where nucleic acid might be released during the process

- Unstable environment for DNA/RNA

inactivation may expose the nucleic acid to an environment where it is not stable e.g. high RNase levels

Direct Comparison Infectivity vs qPCR

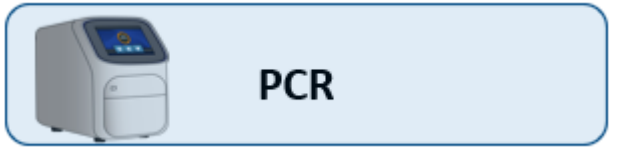
Direct Comparison Infectivity vs qPCR

- A comparison between the bovine infectivity assay (9CFR) and the qPCR bovine testing panel was requested by the US FDA

- Remark:

The 9CFR infectivity test was validated using viruses adapted to grow in tissue culture, not wild type viruses which normally do not grow as efficiently in cell culture systems.

It is likely therefore that the difference in sensitivity presented in this table would be even higher for wild type viruses (i.e. the type of contaminant that might be present in bovine serum).



Direct Comparison Infectivity vs qPCR

Virus	Titre/ml ($TCID_{50}$/ml)	PCR titre/ml (copies/ml)	Particle to infectivity ratio	LOD 9CFR In vitro ($TCID_{50}$/ml)	PCR Titre equivalent of 9CFR LOD (gc/ml)	LOD qPCR Genome Copies/ml	Increase in Sensitivity with qPCR Test
REO	5.4E+07	2.1E+11	3.9E+03	20	7.8E+04	600	129
bPI3	5.0E+08	1.7E+11	3.3E+02	2	6.6E+02	600	1
BPV	3.2E+05	5.6E+10	1.8E+05	2	3.5E+05	100	355
BVDV 1	7.6E+06	1.5E+10	2.0E+03	20	4.1E+04	6 000	7
BAV1	1.6E+05	4.1E+09	2.5E+04	2	5.0E+04	6 000	8
BRSV	6.5E+03	9.5E+09	1.5E+06	2	3.0E+06	600	4919

Abbreviations: REO- Reovirus type 3; bPI3- Bovine parainfluenza virus type 3 ; BPV- Bovine parvovirus; BVDV1- Bovine viral diarrhoea virus type 1; BAV1- Bovine adenovirus type 1; BRSV- Bovine respiratory syncytial virus

Summary and Conclusion

qPCR Testing – Summary

- Very sensitive for a specific virus

Low level viral contaminations are not picked up by the infectivity test, but during production sufficient virus is present to initiate infection. Due to the high particle to infectivity ratio, the qPCR assay can pick up these contaminations

- Support/Replacement of infectivity data

qPCR can provide additional data if an infectivity assay is not possible due to cytotoxicity, matrix interference or insufficient neutralization for viral vaccines

- Positive signal does not mean infectious virus is present

Virus might be inactivated (e.g. gamma irradiation of FBS) but PCR signal remains

Avian cells may contain endogenous components that result in false positive results (e.g. FPERT)

Infectivity Testing – Summary

- Testing for unknown, infectious viral contaminations

Methods are based on the ability grow a wide array of pathogens

- Large amount of inoculum (to increase the sensitivity)

- Can only detect agents that can infect and propagate in indicator cells

Many viruses pathogenic for humans do not infect/replicate readily in culture (e.g. HPV, HCV) or may infect cells but produce no CPE (e.g PCV-1)

- Sensitivity unknown for wild-type strains

Methods are usually established with laboratory-adapted strains

Conclusion

- The advantages and disadvantages from both the infectivity and the qPCR assay have been shown in this presentation
- A risk-based approach and the applicable guidelines should be used to choose the appropriate method
- Depending on the type of sample to be tested, combination of assays might be considered
- Due to the sample matrix, time restraints, limited volume available (e.g. cell or gene therapy products) one method might be preferable to the other
- It is crucial for both methods to include negative and positive controls to ensure that the assay is working properly for the respective sample matrix

Questions?

VIRUSURE

Quality is no coincidence

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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