10. Use of alternative reagents in cell culture

Proteolytic enzyme preparations other than porcine trypsin are available, e.g. recombinant bacterial or plant-derived trypsin, enzymes from invertebrates, bovine trypsin that could be an alternative for use in cell culture. The use of bacterial or plant derived recombinant trypsin minimises in principle the risk for animal virus contamination and the application of such alternatives is therefore encouraged. However, no general recommendation to replace porcine trypsin can currently be given considering that these alternatives need a careful assessment of suitability, quality, sterility and performance characteristics as well as associated risks such as other adventitious agents such as prions from bovine species or invertebrate viruses. When bovine trypsin is used, the bovine virus safety needs to be carefully considered and the Note for Guidance on Minimising the Risk of Transmitting Animal Spongiform Encephalopathy Agents via Human and Veterinary Medicinal Products (EMEA/410/01) in its current version is to be applied.

11. Risk Assessment

This Guideline provides a general quality specification for porcine trypsin, especially with respect to viral safety, and various measures that should be applied during the production of porcine trypsin to minimize the viral risk are described. No combination of the measures outlined below can guarantee complete viral safety, but rather they reduce the risk involved in the use of trypsin in the manufacture of medicinal products. It is therefore necessary for the manufacturer of a medicinal product to take account of this when choosing the trypsin for a particular use by making a risk assessment. The risk assessment should follow the general principles outlined in Ph. Eur. 5.1.7 Viral Safety. Such a risk analysis takes into consideration relevant factors, for example: (1) the epidemiology and control of the animals from which the starting material is sourced (2) the availability of suitable virus test methods and the stage at which such testing is implemented, for instance testing on the animals, production intermediate or final batches of trypsin, or testing at any other stage of production of the medicinal product (3) virus inactivation by trypsin itself, (4) the virus inactivation/removal during manufacture of the trypsin, (5) the stage of manufacture of the medicinal product at which trypsin is used as a reagent, (6) the risk of virus replication in cell cultures used for production of the medicinal product, (7) additional virus inactivation/removal steps applied during the manufacture of the medicinal product, (8) the amount of trypsin to produce a dose of medicinal product, and (9) the route of administration of the medicinal product.

12. Regulatory Aspects

The Marketing Authorisation Holder/Applicant of the medicinal product should have sufficient information on the trypsin to allow a comprehensive risk assessment and provide a sufficient data package to the competent authority for assessment. This should include a description of testing methods and the stage at which virus testing is performed, as well as the volumes and sensitivity of the virus tests. Study reports validating virus reduction steps should be provided according to Guideline CPMP/BWP/268/95. In the case of a change of supplier of trypsin, data as outlined above should be provided for the new trypsin.

This guideline is for prospective implementation, i.e. for new marketing applications. However it is advisable to consider the risk of contamination from porcine trypsin already at early stages of product development. It is recognised that it may take some time to implement the recommendations in this guideline, therefore, a transition period of a maximum of two years from the date of adoption of this guideline is set. Nevertheless, in the interim phase, a re-assessment of the virus safety is

recommended for authorised live virus vaccines and cell based medicinal products that use porcine trypsin and do not incorporate viral inactivation/removal steps in the manufacturing process.

References

EMEA/P/24143/2004 Rev. 1 corr. Procedure for European Union Guidelines and related documents within the pharmaceutical legislative framework.

EMA/732522/2010 European Medicines Agency confirms that presence of unexpected viral DNA in live attenuated vaccines does not raise public health concerns. http://www.ema.europa.eu/docs/en_GB/document_library/Press_release/2010/11/WC500099132.pdf

Lackner C, Leydold SM, Modrof J, Farcet MR, Grillberger L, Schaefer B, Anderle H, Kreil TR. Reduction of spiked porcine circovirus during the manufacture of a Vero cell-derived vaccine Vaccine, in press.

Marcus-Sekura C, Richardson JC, Harston RK, Dane N. Sheets RL. Evaluation of the human host range of bovine and porcine viruses that maycontaminate bovine serum and porcine trypsin used in the manufacture of biological products. Biologicals 39:359-369, 2011.

Yang et al. et al 2013 *PDA Europe: Virus and TSE Safety Forum.*

Melnick and Wallis. 1977. Problems related to the use of serum and trypsin in the growth of monkey kidney cells. Dev. Biol. Stand 37:77-82.

Victoria JG, Wang C, Jones MS, Jaing C, McLoughlin K, Gardner S, Delwart EL. Viral nucleic acids in live-attenuated vaccines: detection of minority variants and an adventitious virus. *J Virol*. 2010 Jun;84(12):6033-40

WHO recommendations for the evaluation of animal cell cultures as substrates for the manufacture of biological medicinal products and for the characterization of cell banks (2010).

Note for guidance on virus validation studies: the design, contribution and interpretation of studies validating the inactivation and removal of viruses (CPMP/BWP/268/95).

Note for guidance on the use of bovine serum in the manufacture of human biological medicinal products (CPMP/BWP/1793/02).

European Pharmacopoeia - General text 5.2.3 on cell substrates for the production of vaccines for human use.

European Pharmacopoeia Monograph 2262 Bovine Serum.

European Pharmacopoeia 2.6.7 Mycoplasmas.

European Pharmacopoeia general chapter 5.1.7 Viral Safety.

European Pharmacopoeia Monograph (0694) Trypsin.

U.S. Food and Drug Administration
Protecting and Promoting Public Health
www.fda.gov

FDA's Approach to Adventitious-Agent Testing of Cell Substrates and Viral Vaccines: Traditional and Novel Methods

Keith Peden
Division of Viral Products
OVRR, CBER, FDA
May 7, 2010

Outline of Talk

- Approaches for the detection of adventitious agents
- Current recommendations for adventitious agent testing
  - Non-specific *in vivo* tests
  - Non-specific *in vitro* tests
  - Non-specific biochemical/molecular tests
  - Specific molecular tests
- Novel molecular technologies for adventitious-agent
- detection
- Issues associated with each
- Regulatory challenges

Vaccines: General Comments

- Vaccines are the most effective way to control infectious diseases
- The safety record of vaccines is excellent
- Maintenance of the public confidence in vaccines is critical to public health

Definition of an Adventitious Agent

- Adventitious agents are microorganisms that have been unintentionally introduced into the manufacturing process of a biological product
- Include bacteria, fungi, mycoplasmas, rickettsia, protozoa, parasites, TSE agents, and viruses

Adventitious Agent Detection Issues

- For a substance to be considered 'free' of an adventitious agent, assays must demonstrate that a defined quantity of a vaccine is negative for that agent at a defined level of sensitivity
- The level of assay sensitivity is determined experimentally using standardized reagents
- Alternatively, a validated manufacturing process shown to remove an adventitious agent to a defined level may be used to demonstrate freedom from that agent

Approaches for the Detection of Adventitious Agents

- Adventitious agents are detected by a combination of methods and strategies and at various stages of production
- Reliance is not placed on a single approach or method
- Multiple strategies provide, to the extent possible, assurance that products are 'free' from adventitious agents
- Manufacturing process (cGMP) should result in high quality vaccines

Current FDA Recommendations For Cell Substrates

- Characterization and Qualification of Cell Substrates and Other Biological Starting Materials Used in the Production of Viral Vaccines for the Prevention and Treatment of Infectious Diseases
- http://www.fda.gov/downloads/biologicsbloodvaccines/guidancecomplianceregulatoryinformation/guidances/vaccines/ucm202439.pdf

Current Methods of Detection of Adventitious Agents

- Broad, overlapping schemes to detect as wide an array of viruses as possible
- Methods evolved over time; in many ways recapitulate the history of virus discovery
- Infectivity assays in animals and cell culture
- Non-specific methods – known/unknown agents
  - *in vivo* (animals)
  - *in vitro* (cell culture)
  - molecular/biochemical
- Specific methods – known agents
  - molecular, *e.g.*, PCR

Non-Specific Methods: *In Vivo* Systems

- Various animal models used, *e.g.*, adult mice, suckling mice, embryonated hens' eggs, guinea pigs, rabbits
- These tests originally used because they detected viruses not readily detected in other systems

Non-Specific Methods: *In Vivo* Systems

Examples of viruses detected in mice include:
cocksackie A & B viruses; picornaviruses (polioviruses, echoviruses); alphaviruses; bunyaviruses (phleboviruses, nairoviruses); arenaviruses; flaviviruses; rabies virus; herpesviruses; lymphocytic choriomeningitis virus; also various murine viruses

Examples of viruses detected in eggs include:
orthomyxoviruses; paramyxoviruses; alphaviruses; vesiculoviruses; herpesviruses; poxviruses; rhabdoviruses

Limitations of *In Vivo* Tests

- Sensitivity is unknown for wild-type strains, as methods were usually established with laboratory-adapted strains
- Many viruses pathogenic for humans do not infect or replicate in rodents or eggs

Antibody-Production Tests

- Test article is inoculated into animals, and an adventitious agent is detected by the presence of antibodies to that agent
- Mainly performed on cell substrates when there is a possibility of exposure to rodent agents

Antibody-Production Tests

Viruses detected include:

ectromelia virus, mouse rotavirus, Hantaan virus, Lymphocytic choriomeningitis virus, lactate dehydrogenase virus, minute virus of mice; mouse adenovirus, murine cytomegalovirus, mouse encephalomyelitis virus, mouse hepatitis virus, pneumonia virus of mice, polyoma virus, reovirus type 3, Sendai virus, thymic virus, K virus, simian virus 5, mouse encephalomyelitis virus, Hantaan virus, Kilham rat virus, mouse adenovirus, rat coronavirus, Toolan virus, sialdacryoadenitis virus

Non-Specific Tests: *In Vitro* Systems

Non-Specific Tests: *in vitro* Systems

- Methods are based on the ability of cell cultures to grow a wide array of pathogens; also based on their extensive use in diagnostic laboratories to detect human pathogens
- Large amount of inocula can be applied, thus increasing their sensitivity
- Cell-culture tests can detect a variety of adventitious viruses, including cytopathic viruses, hemadsorbing viruses, and hemagglutinating viruses
- Selection of the cell line depends upon the potential exposure to agents (species and tissue type of cell substrate; human diploid cells; monkey kidney cells)

Cell-Culture Method: Readout

- Evidence of virus contamination indicated by:
- Cytopathic effects in culture
- For non-cytopathic viruses, test at the end of the observation period for:
  - hemadsorption (binding of red blood cells from human, rhesus macaque, guinea pig, chicken)
  - hemagglutination (agglutinate red blood cells)
- Other readouts can be used, such as antibody staining, PCR, *etc.*

Limitations of *In Vitro* Tests

- Only can detect agents that can infect and propagate in indicator cells
- Sensitivity is unknown for wild-type strains, as methods were usually established with laboratory-adapted strains
- Many viruses pathogenic for humans do not infect or replicate readily in culture (*e.g.*, HPV, HCV)

Other Non-Specific Tests

Transmission Electron Microscopy

- Can detect virus particles in a cell substrate, including those from endogenous viruses
- Morphology provides indication of the type of viral contaminant
- Insensitive assay – generally considered to require 106 particles per mL to be detected
- Qualitative assay; a positive result would require additional tests (*e.g.*, PCR, infectivity)

Reverse Transcriptase (RT) Assay for Retrovirus Detection

- All retroviruses have RT in their virions; therefore, these assays can detect all retroviruses
- Quantitative PERT assays are now recommended
- Some cell substrates express non-infectious endogenous retroviral particles, *e.g.*, eggs, chick embryo fibroblasts, CHO cells
- Because of high assay sensitivity, false positive signals can be obtained from cell lysates
- Positive result may require infectivity assays

Specific Tests for Viruses

21

PCR Tests

- When viruses are of concern in a specific product, additional testing is recommended
- Such tests are based on the virus sequence
- Conventional PCR and qPCR
- Partially degenerate primer PCR that detects members of a virus family
- Examples:
  - Human pathogens in certain human cells: HIV, HCV, *etc.*
- When warranted, PCR tests for various animal viruses are recommended

22

Novel Molecular Methods for Adventitious Agent Detection

Strategy for Adventitious Agent Detection

Sample selection
- Cellular genome (DNA)
- Transcriptome (RNA)
- Virus particle

- Amplification schemes
  - Family-specific primers
  - Degenerate primers, *etc.*
- Detection methodologies
  - Mass spectrometry
  - Microarray
  - High-throughput sequencing

24

Sample Selection

Cellular genomic DNA
- Advantage: all sequences represented
- Disadvantages: complexity is high
  insensitivity of virus detection

- Transcriptome (RNA)
  - Advantages: all transcribed sequences represented
    lower sequence complexity
    increased sensitivity of virus detection
  - Disadvantage: results are cell-cycle dependent
    endogenous viral sequences expressed
- Virus particle
  - Advantages: enrichment for viral sequences lowers complexity
    increased sensitivity of virus detection
  - Disadvantage: might exclude some viral sequences in fragile capsids

25

Amplification Schemes

- PCR using primers to amplify members of a virus family
  - Advantage: should amplify most known viruses
  - Disadvantage: unknown viruses will be missed
- PCR using fully degenerate primers or anchored degenerate primers
  - Advantage: all virus sequences should be amplified
  - Disadvantage: detection and identification might be complex

26

Detection Methodologies - 1

- Mass spectrometry
  - Only can detect known viruses or virus families
  - Sensitivity not established
- Microarray methods
  - Direct application of nucleic acids to microarray
    - Considerations: Low sensitivity, but no introduction of sequence bias
  - PCR amplification prior to application of nucleic acids to microarray
    - Considerations: Increased sensitivity, but potential for introduction of sequence bias and contamination

27

Detection Methodologies – 2

- High-throughput sequencing
  - Several technologies currently available
  - New bioinformatics tools required to analyze vast quantities of data generated; usually not available in house
  - Expertise, curated databases, standardized analysis methods and programs required
  - Can be applied to genomes, transcriptomes, virus particles
  - Interpretation can be unclear:
    - Cellular genomes: plenty of sequence that might be difficult to analyze
    - Transcriptomes: likely to get large amounts of endogenous retroviral sequence; unclear how to interpret this
    - Virus particles: likely to produce fewer sequences, but also could get uninterpretable results
- Results may become interpretable with time

Considerations With New Generation Molecular Methods

- Sensitivities usually not determined
- Do not indicate whether virus is infectious
- Each different method may require different types of standardization and standards to be used in a regulatory context
- Breadth of detection not studied
- Reproducibility or robustness generally not known
- Many techniques are not commercially available
- Many results will require follow up

Final Considerations

Adventitious Agents: A Continuing Challenge

- Novel viruses are being discovered and will continue to be discovered
- Many of these could be present as adventitious agents of cell substrates or biological products
- New cell substrates from insects, plants, fungi, *etc.* will bring additional issues

Summary

- Adventitious agent detection relies on the use of multiple overlapping strategies
- Detection methods for adventitious agents continue to evolve and represent improvements in technology, including sensitivity and throughput
- These new technologies can potentially be a powerful means to support safe product development

Regulatory Challenges

- Evaluation of risk from new viruses detected using these evolving technologies may be complex
  - No infectivity assays for novel viruses
  - Pathogenicity of viruses often takes years to establish
- Issues in use of assays
  - Selection of assay
  - Standardization of each assay
  - Validation of assays
- Harmonization among various National Regulatory Authorities

Acknowledgements

Phil Krause
Andrew Lewis
Arifa Khan
Robin Levis
Jerry Weir
Kostya Chumakov
Marion Gruber
Norman Baylor

DISCUSSION POINTS

- Please discuss the application of emerging technologies, and the implications for their use in the detection of known and unknown adventitious agents in vaccines currently licensed, as well as those under development