Application of Next Generation Sequencing to Biological and Biotechnological Products: How to Balance Regulation and Innovation

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❑ Outline

- Viral safety
- Applications of NGS in biological products
- Validation requirements for NGS
- Conclusion and Future Perspective

- Safety of vaccines and other biological products is critical.

- Safety is particularly critical for:

  - live vaccines
  - gene therapy viral vectors
  - cell therapy medicinal products

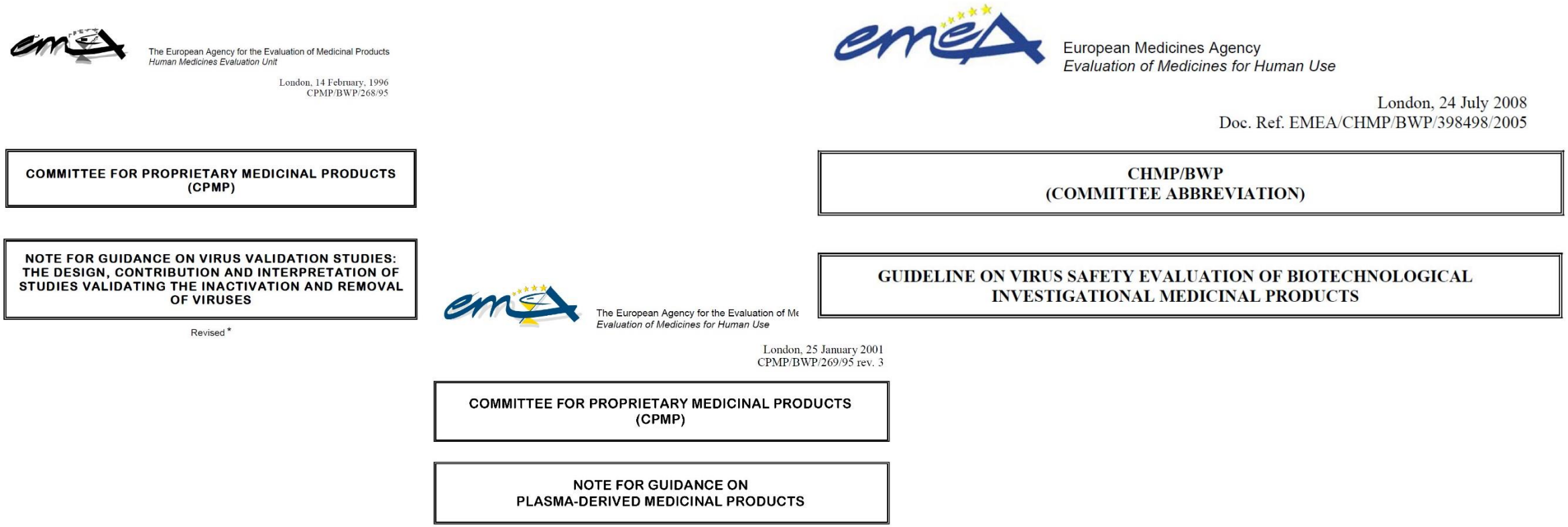
ICH HARMONISED TRIPARTITE GUIDELINE

VIRAL SAFETY EVALUATION OF BIOTECHNOLOGY PRODUCTS DERIVED FROM CELL LINES OF HUMAN OR ANIMAL ORIGIN

Q5A(R1)

- ICH Q5A, specifically requires that a manufacturer of biological products for human use demonstrate the capability of the manufacturing process to remove or inactivate known contaminants.

- Various EMA guidelines provide recommendations for validation of viral inactivation biopharmaceutical products.



- These recommendations also set specific values for virus clearance levels that had to be attained.

❑ Detection of viral contaminants in biopharmaceutical products

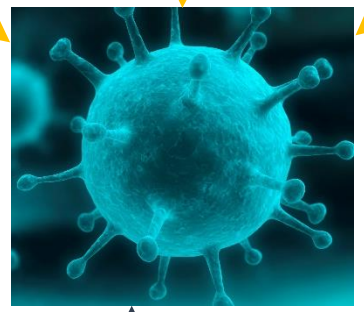
Non-specific test methods

Cell cultures

Infectivity Assay
(Retroviruses)

Tests in animals

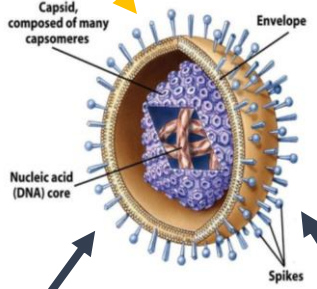
Live Infectious virus



9CFR for Bovine & Porcine viruses

Test for reverse transcriptase

Transmission Electron Microscopy (TEM)



Viral components

PCRs

in vivo Antibody production tests (MAP, RAP, HAP)

Virus-specific test methods

❑Original concerns focused on a relatively small number of known viruses associated with the production cell lines.

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Viral Nucleic Acids in Live-Attenuated Vaccines: Detection of Minority Variants and an Adventitious Virus▽†

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Metagenomics and a panmicrobial microarray were used to examine eight live-attenuated viral vaccines. Viral nucleic acids in trivalent oral poliovirus (OPV), rubella, measles, yellow fever, varicella-zoster, multivalent measles/mumps/rubella, and two rotavirus live vaccines were partially purified, randomly amplified, and pyrosequenced. Over half a million sequence reads were generated covering from 20 to 99% of the attenuated viral genomes at depths reaching up to 8,000 reads per nucleotides. Mutations and minority variants, relative to vaccine strains, not known to affect attenuation were detected in OPV, mumps virus, and varicella-zoster virus. The anticipated detection of endogenous retroviral sequences from the producer avian and primate cells was confirmed. Avian leukosis virus (ALV), previously shown to be noninfectious for humans, was present as RNA in viral particles, while simian retrovirus (SRV) was present as genetically defective DNA. Rotarix, an orally administered rotavirus vaccine, contained porcine circovirus-1 (PCV1), a highly prevalent nonpathogenic pig virus, which has not been shown to be infectious in humans. Hybridization of vaccine nucleic acids to a panmicrobial microarray confirmed the presence of endogenous retroviral and PCV1 nucleic acids. Deep sequencing and microarrays can therefore detect attenuated virus sequence changes, minority variants, and adventitious viruses and help maintain the current safety record of live-attenuated viral vaccines.

Case study: PCV in Rotavirus Vaccine

Case study: Sf Rhabdovirus

JVI Journals.ASM.org

Identification of a Novel Rhabdovirus in *Spodoptera frugiperda* Cell Lines

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ABSTRACT

The Sf9 cell line, derived from *Spodoptera frugiperda*, is used as a cell substrate for biological products, and no viruses have been reported in this cell line after extensive testing. We used degenerate PCR assays and massively parallel sequencing (MPS) to identify a novel RNA virus belonging to the order *Mononegavirales* in Sf9 cells. Sequence analysis of the assembled virus genome showed the presence of five open reading frames (ORFs) corresponding to the genes for the N, P, M, G, and L proteins in other rhabdoviruses and an unknown ORF of 111 amino acids located between the G- and L-protein genes. BLAST searches indicated that the *S. frugiperda* rhabdovirus (Sf-rhabdovirus) was related in a limited region of the L-protein gene to Taastrup virus, a newly discovered member of the *Mononegavirales* from a leafhopper (Hemiptera), and also to plant rhabdoviruses, particularly in the genus *Cytorhabdovirus*. Phylogenetic analysis of sequences in the L-protein gene indicated that Sf-rhabdovirus is a novel virus that branched with Taastrup virus. Rhabdovirus morphology was confirmed by transmission electron microscopy of filtered supernatant samples from Sf9 cells. Infectivity studies indicated potential transient infection by Sf-rhabdovirus in other insect cell lines, but there was no evidence of entry or virus replication in human cell lines. Sf-rhabdovirus sequences were also found in the Sf21 parental cell line of Sf9 cells but not in other insect cell lines, such as BT1-TN-$_5$B$_{1-4}$ (Tn$_5$; High Five) cells and Schneider's Drosophila line 2 [D.Mel.(2); SL2] cells, indicating a species-specific infection. The results indicate that co[illegible] methods may be complemented by state-of-the-art technologies with extensive bioinformatics analysis for identificati[illegible] novel viruses.

❑ Limitation of conventional assays

- Cell lines may not be permissive for the virus
- Virus replication is not visible (no CPE)
- Cytotoxicity – Neutralization - Interference
- Virus not detected by PCR primers

- Regulatory expectations on viral safety of biopharmaceutical products have evolved over the past decade.
- Today, the concerns are much broader, encompassing unknown and uncharacterized agents.
- Increasingly stringent conditions are intended to decrease the risk of transmitting viruses.
- Next generation sequencing (NGS) is a sensitive and un-biased detection method for adventitious agents.

Evolution of European Pharmacopoeia

- Ph. Eur. Chapter 5.2.14: "Substitution of *in vivo* method(s) by *in vitro* method(s) for the quality control of vaccines", implemented 1/2018, version 9.3

5.2.14. SUBSTITUTION OF *IN VIVO* METHOD(S) BY *IN VITRO* METHOD(S) FOR THE QUALITY CONTROL OF VACCINES

PURPOSE

The purpose of this general chapter is to provide guidance to facilitate the implementation of *in vitro* methods as substitutes for existing *in vivo* methods, in cases where a typical one-to-one assay comparison is not appropriate for reasons unrelated to the suitability of one or more *in vitro* methods. This general chapter will not discuss the details of assay validation as such, since those principles are described elsewhere.

The general chapter applies primarily to vaccines for human or veterinary use, however the principles described may also apply to other biologicals such as sera.

Detection of viral extraneous agents by novel molecular methods

Detection of viral extraneous agents in cell banks, seed lots and cell culture harvests is currently conducted using a panel of *in vivo* and *in vitro* methods at different stages of the manufacturing process. Novel, sensitive molecular techniques with broad detection capabilities are available, including deep sequencing or high-throughput sequencing methods, degenerate polymerase chain reaction (PCR) for whole virus families or random-priming methods (associated or not with sequencing), hybridisation to oligonucleotide arrays and mass spectrometry. The use of these new molecular methods has highlighted gaps in the existing testing strategy by identifying previously undetected viral contaminants in final product, the cell banks from which it was produced and intermediate manufacturing stages. These new molecular methods (e.g.

Evolution of European Pharmacopoeia

- Ph. Eur. Chapter 5.2.3: “Cell Substrates for the production of vaccines for human use”, version 9:0 and updated version 9.3

5.2.3. CELL SUBSTRATES FOR THE PRODUCTION OF VACCINES FOR HUMAN USE

This general chapter deals with diploid cell lines and continuous cell lines used as cell substrates for the production of vaccines for human use; additional issues specifically related to vaccines prepared by recombinant DNA technology are covered by the monograph *Products of recombinant DNA technology (0784)*. The testing to be carried out at the various stages (cell seed, master cell bank (MCB), working cell bank (WCB), end of productions cells (EOPC) or extended cell bank (ECB) corresponding to cells at or beyond the maximum population doubling level used for production) is indicated in Table 5.2.3.-1. General provisions for the use of cell lines and test methods are given below. Where primary cells or cells that have undergone a few passages without constitution of a cell bank are used for vaccine production, requirements are given in the individual monograph for the vaccine concerned.

Infectious extraneous agents. For cell lines for vaccine production, the testing for infectious extraneous agents must be carried out based on a risk assessment. The origin of the cell substrate as well as the potential extraneous agents that may be inadvertently introduced during production processes or through the use of animal or plant derived raw materials must be taken into account in the choice of suitable permissive cells. One such strategy is given in Table 5.2.3.-1, but alternative strategies could focus on more extensive testing at the MCB or WCB level. In any case, any strategy must be justified and lead to the same level of safety as outlined in Table 5.2.3.-1. New, sensitive molecular techniques with broad detection capabilities are available, including massive parallel sequencing (MPS) methods, degenerate polymerase chain reaction (PCR) for whole virus families or random-priming methods (associated or not with sequencing), hybridisation to oligonucleotide arrays and mass spectrometry. These methods may be used either as an alternative to *in vivo* or specific NAT tests or as a supplement/alternative to *in vitro* culture tests, in agreement with the competent authority. The capacity of the process to remove/inactivate specific viruses must take into account the origin and culture history of the cell line and adventitious viruses that are known to persistently infect the species of origin, for example, simian virus 40 in rhesus monkeys, Flock house virus in insect cells or viruses that may inadvertently be introduced during production processes or through the use of raw materials of animal or plant origin. For cell lines of insect origin, tests for specific

Evolution of European Pharmacopoeia

- Ph. Eur. Chapter 2.6.16: "Tests for extraneous agents in viral vaccines for human use", version 10.2

2.6.16. TESTS FOR EXTRANEOUS AGENTS IN VIRAL VACCINES FOR HUMAN USE

INTRODUCTION

A strategy for testing extraneous agents in viral vaccines must be developed based on a risk assessment following the principles of viral contamination risk detailed in general chapter *5.1.7. Viral safety*. This strategy includes a full package of suitable tests that are able to detect different families of extraneous agents that may infect the source of virus strains including cell substrates and raw material of animal or plant origin. It also takes into account the capacity of the manufacturing process to remove or inactivate viruses. The

New, sensitive molecular methods with broad detection capabilities are available. These new approaches include high-throughput sequencing (HTS) methods, nucleic acid amplification techniques (NAT) (e.g. polymerase chain reaction (PCR), reverse transcriptase PCR (RT-PCR), product-enhanced reverse transcriptase (PERT) assays) for whole virus families or random-priming methods (associated or not with sequencing), hybridisation to oligonucleotide arrays, and mass spectrometry with broad-spectrum PCR. These methods may be used either as an alternative to *in vivo* tests and specific NAT or as a supplement/alternative to *in vitro* culture tests based on the risk assessment and with the agreement of the competent authority.

WHO Focus on NGS

WHO - TRS 978, ECBS 2010:

"New, sensitive, molecular methods, with broad detection capabilities are being developed... The new generation of massively parallel (deep) sequencing (MPS) methods may have particular utility. They can be applied to detect virions after nuclease treatment to remove cellular DNA and unencapsidated genomes. Used in this mode, MPS has been used to discover new viruses in serum and other tissues and has revealed the contamination of human vaccines by porcine circovirus."

"MPS can also be employed to screen cell substrates for both latent and lytic viruses by sequencing the transcriptome. In this mode, enormous quantities of data are generated, and robust bioinformatic methods are required to detect viral sequences by either positive selection against viral databases or negative selection to remove cellular sequences."

WHO - TRS 878, Annex 1

"It is probable that application of methods of this type will be expected or required by regulatory agencies in future."

WHO TRS 993 Annex 2. Scientific principles for regulatory risk evaluation on finding an adventitious agent in a marketed vaccine

"WHO defined ***Next-generation sequencing (NGS) as "****high-throughput sequencing technology that processes sequences in parallel, producing thousands or millions of sequences at once from a sample… Significant bioinformatics using curated (trusted) databases are needed to analyze the considerable amount of data generated in each sequencing run."*

"New methods and technologies, such as NGS or microarrays, are powerful tools for the detection and identification of sequences from viruses and other adventitious agents without prior knowledge of the nature of the agent. In the future such new technologies may uncover the presence of other, as yet unrecognized, adventitious agents."

❑ Application and Usefulness of NGS -1

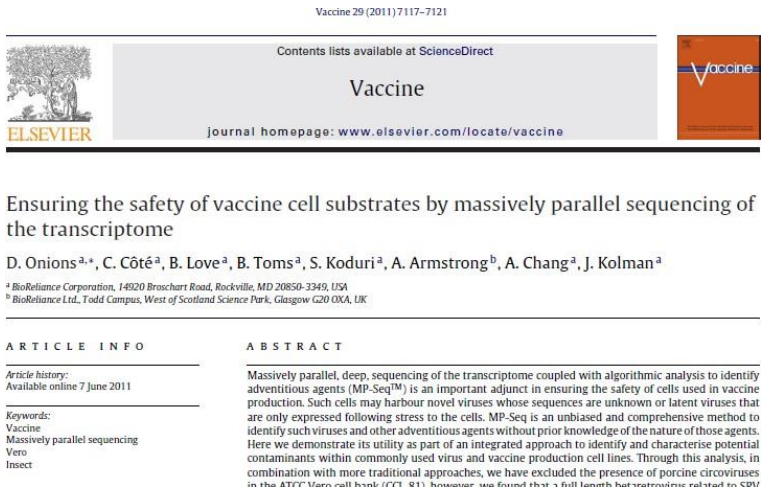
- Detection method for adventitious agents.

Removal, supplementation, replacement, substitution of in vivo adventitious agent tests

Substitution of in vitro nucleic acid based tests

❑ Application and Usefulness of NGS -2

➢ Characterization, screening studies



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Contents lists available at ScienceDirect

Vaccine

journal homepage: www.elsevier.com/locate/vaccine

Ensuring the safety of vaccine cell substrates by massively parallel sequencing of the transcriptome

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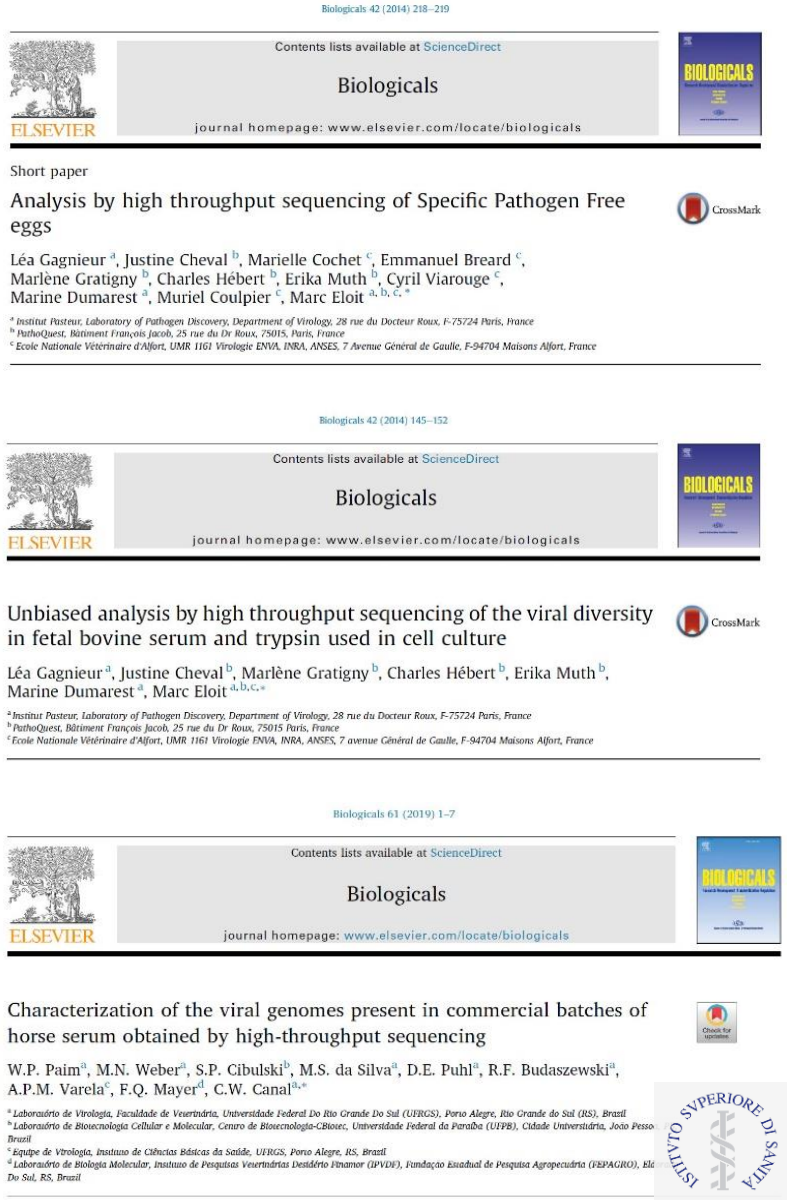
ABSTRACT

Massively parallel, deep, sequencing of the transcriptome coupled with algorithmic analysis to identify adventitious agents (MP-Seq™) is an important adjunct in ensuring the safety of cells used in vaccine production. Such cells may harbour novel viruses whose sequences are unknown or latent viruses that are only expressed following stress to the cells. MP-Seq is an unbiased and comprehensive method to identify such viruses and other adventitious agents without prior knowledge of the nature of those agents. Here we demonstrate its utility as part of an integrated approach to identify and characterise potential contaminants within commonly used virus and vaccine production cell lines. Through this analysis, in combination with more traditional approaches, we have excluded the presence of porcine circoviruses

Pre Master Cell Bank / Pre Seed

Raw Materials



Biologicals 42 (2014) 218–219

Contents lists available at ScienceDirect

Biologicals

journal homepage: www.elsevier.com/locate/biologicals

Short paper

Analysis by high throughput sequencing of Specific Pathogen Free eggs

Léa Gagnieur[a], Justine Cheval[b], Marielle Cochet[c], Emmanuel Breard[c], Marlène Gratigny[b], Charles Hébert[b], Erika Muth[b], Cyril Viarouge[c], Marine Dumarest[a], Muriel Coulpier[c], Marc Eloit[a,b,c,*]

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Biologicals 42 (2014) 145–152

Contents lists available at ScienceDirect

Biologicals

journal homepage: www.elsevier.com/locate/biologicals

Unbiased analysis by high throughput sequencing of the viral diversity in fetal bovine serum and trypsin used in cell culture

Léa Gagnieur[a], Justine Cheval[b], Marlène Gratigny[b], Charles Hébert[b], Erika Muth[b], Marine Dumarest[a], Marc Eloit[a,b,c,*]

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Biologicals 61 (2019) 1–7

Contents lists available at ScienceDirect

Biologicals

journal homepage: www.elsevier.com/locate/biologicals

Characterization of the viral genomes present in commercial batches of horse serum obtained by high-throughput sequencing

W.P. Paim[a], M.N. Weber[a], S.P. Cibulski[b], M.S. da Silva[a], D.E. Puhl[a], R.F. Budaszewski[a], A.P.M. Varela[c], F.Q. Mayer[d], C.W. Canal[a,*]

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Do Sul, RS, Brazil

❑ Application and Usefulness of NGS -3

- Investigational tool

For example: to clarify if an identified contaminant is replicative

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Biologicals

journal homepage: www.elsevier.com/locate/biologicals

Use of a new RNA next generation sequencing approach for the specific detection of virus infection in cells

Audrey Brussel[a], Kerstin Brack[b], Erika Muth[c], Rudolf Zirwes[c], Justine Cheval[c], Charles Hebert[c], Jean-Marie Charpin[c], Alice Marinaci[b], Benoit Flan[a], Horst Ruppach[b], Pascale Beurdeley[c], Marc Eloit[c,d,e,*]

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RESEARCH ARTICLE
Applied and Environmental Science

Adventitious Virus Detection in Cells by High-Throughput Sequencing of Newly Synthesized RNAs: Unambiguous Differentiation of Cell Infection from Carryover of Viral Nucleic Acids

Justine Cheval,[a] Erika Muth,[a] Gaëlle Gonzalez,[b] Muriel Coulpier,[b] Pascale Beurdeley,[a] Stéphane Cruveiller,[a] Marc Eloit[a,c,d]

❑ Other potential applications of NGS

- NGS could also be used at different stages e.g. product development, manufacturing or finished product:
  - Identification and characterization of vaccine strains
  - Evaluation of genetic stability of vaccine strains after successive passages
  - Reversion to virulence of the attenuated vaccine strains

NGS Platforms mostly used

Short reads

illumina

iontorrent
by Thermo Fisher Scientific

MGI 华大智造

Single molecule

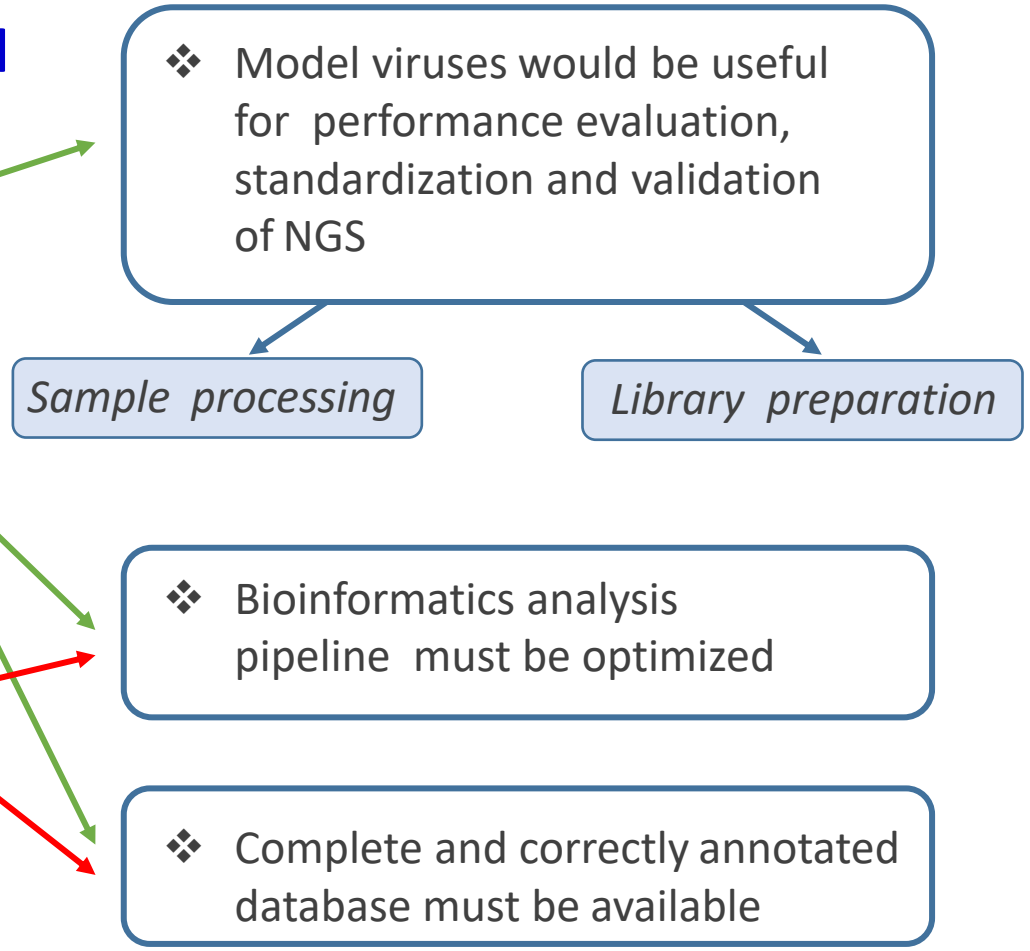
PACIFIC BIOSCIENCES®

Oxford NANOPORE Technologies

❑ Challenge to Use NGS to Detect Adventitious Agents

Major challenge → Validation of NGS Method

- Diversity of viral targets and biological matrices (e.g. cell banks, viral seeds, raw materials)
- Complexity of the NGS technologies and associated bioinformatics



❑ Due to the need to validate each step of NGS method, a coordinate work among specialists is important

In 2014 the Advanced Virus Detection Technologies Interest Group (AVDTIG), gathering together Regulatory and Government agencies, Industry, Service providers, Technology developers, and Academics from all over the world, has been formed.

Efforts of the Advanced Virus Detection Technologies Interest Group (AVDTIG) for NGS applications in Biologics

2nd Conference on Next Generation Sequencing for Adventitious Virus Detection in Human and Veterinary Biologics - An IABS-EU Meeting

November 13-14, 2019 - Het Pand, University of Ghent, Belgium

Jean-Pol Cassart and AVDTIG

❑ Validation and Standardization -1

➢ Preliminary consideration

- ❖ NGS is not a quantitative analysis
- ❖ Sample flow similar to PCR assays

➢ Sample and library preparation

- ❖ Extractions and recovery of viral nucleic acids controls (accuracy of the method)
- ❖ Extractions and recovery

❑ Validation and Standardization -2

- Appropriate model viruses for spiking studies (needs for a standard).

  - Efficiency of the different steps of the methodology
  - Evaluation of total NGS workflow in different biological matrices
  - Compare NGS with current assays for virus detection (PCR, *in vivo*, *in vitro*)
  - Generation of well-characterized datasets for evaluating bioinformatics pipelines
  - Sensitivity studies

Reagent available from NIBSC catalogue
www.nibsc.org/products ref: 11/242-001

Vaccine 34 (2016) 2035–2043

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Vaccine

journal homepage: www.elsevier.com/locate/vaccine

Development of a candidate reference material for adventitious virus detection in vaccine and biologicals manufacturing by deep sequencing

Edward T. Mee[a,*], Mark D. Preston[b], CS533 Study Participants[1], Philip D. Minor[a], Silke Schepelmann[a]

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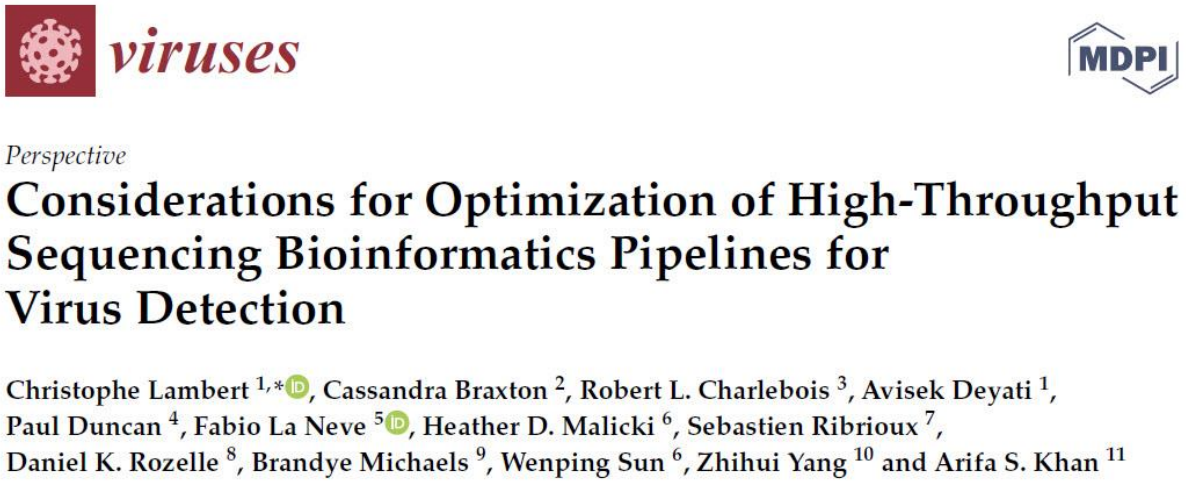
❑ Validation and Standardization -3

- Specificity
  - Demonstrated by a negative control extracted and sequenced in parallel
  - Breadth of detection confirmation

❑ Validation and Standardization -4

➢ Bioinformatics - Pipeline optimization

- ❖ Criteria for acceptable quality of reads
- ❖ Parameters for short read assembly
- ❖ hybrid assembly to correct high error-rate in long-read sequencing
- ❖ Strategies to identify novel viruses with minimal similarity to known sequences



viruses

MDPI

Perspective

Considerations for Optimization of High-Throughput Sequencing Bioinformatics Pipelines for Virus Detection

Christophe Lambert [1,*], Cassandra Braxton [2], Robert L. Charlebois [3], Avisek Deyati [1], Paul Duncan [4], Fabio La Neve [5], Heather D. Malicki [6], Sebastien Ribrioux [7], Daniel K. Rozelle [8], Brandye Michaels [9], Wenping Sun [6], Zhihui Yang [10] and Arifa S. Khan [11]

❑ Validation and Standardization -5

- Development of a complete and correctly annotated, publicly available, Reference Virus Database

Database available at:

https://rvdb.dbi.udel.edu/

RESEARCH ARTICLE
Applied and Environmental Science

A Reference Viral Database (RVDB) To Enhance Bioinformatics Analysis of High-Throughput Sequencing for Novel Virus Detection

Norman Goodacre,[a] Aisha Aljanahi,[a]* Subhiksha Nandakumar,[a] Mike Mikailov,[b] Arifa S. Khan[a]

❑ NGS positive sample: follow-up strategy

- Confirmation of a “true” hit
  - Can the results be confirmed by PCR or another assay?
  - Is a complete viral genome present?
- Determination of biological relevance and significance of a positive signal
  - Are particles present?
  - Are the particles infectious?
  - Is there a replication-competent virus?
  - Can the nucleic acid/particles be quantified?

❑ Summary of the steps which need validation to utilize NGS for Biological and Biotechnological Products -1

- **Sample preparation and processing**
  - Extraction efficiency of different virus structure (with/out envelope)
  - cDNA synthesis of different virus genome (Single/double strand; DNA/RNA)
  - Library preparation
  - Enrichment steps for viral nucleic acid Controls (reagents, method)
- **Sequencing platform**
  - Selection of sequencer to provide sufficient reads to detect a low level virus
  - Consider error rate of sequencing technology: short reads vs long reads

❑ Summary of the steps which need validation to utilize NGS for Biological and Biotechnological Products -2

- **Bioinformatics**
  - Strategies for detection of known and novel viruses (nucleotide vs amino acids, programs/tools, reads vs contigs, criteria and parameters for runs)
  - Databases
  - Unmapped reads?
  - Re-analysis?

❑ Conclusions

- Evaluation of NGS platforms for virus detection
- Standardization of the methods, including availability of virus references representing different virus families
- Developing bioinformatics tools and strategies for accurate virus detection and data interpretation

❑ Future perspective -1

- Further dialogue between researchers, developers, companies and regulators to understand **current hurdles** to approve the implementation of NGS.

- Improvement of experimental projects for an accurate standardization of all the steps involved in NGS and biologicals control.

- Collaboration between researchers, companies and regulators for the development of specific guidance on requirements for regulatory acceptance of NGS.

❑ Future perspective -2

- Coordination between regulatory bodies to **harmonize requirements**

- Organize collaborative studies to address technology complexity on common grounds

❑How to Balance Regulation and Innovation

- Absence of specific guideline
- Next Generation Sequencing could be accepted
- A strict Validation is requested
- Validation must cover all the steps
- To be easily usable by Assessors, all Validation steps must have a unique rationale

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Istituto Superiore di Sanità – Rome - Italy