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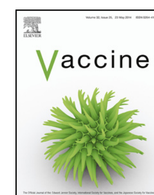

Development of a candidate reference material for adventitious virus detection in vaccine and biologicals manufacturing by deep sequencing

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ABSTRACT

Background: Unbiased deep sequencing offers the potential for improved adventitious virus screening in vaccines and biotherapeutics. Successful implementation of such assays will require appropriate control materials to confirm assay performance and sensitivity.
Methods: A common reference material containing 25 target viruses was produced and 16 laboratories were invited to process it using their preferred adventitious virus detection assay.
Results: Fifteen laboratories returned results, obtained using a wide range of wet-lab and informatics methods. Six of 25 target viruses were detected by all laboratories, with the remaining viruses detected by 4–14 laboratories. Six non-target viruses were detected by three or more laboratories.
Conclusion: The study demonstrated that a wide range of methods are currently used for adventitious virus detection screening in biological products by deep sequencing and that they can yield significantly different results. This underscores the need for common reference materials to ensure satisfactory assay performance and enable comparisons between laboratories.

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1. Introduction

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Production of live viral vaccines on animal cell or egg substrates carries the risk of adventitious virus contamination of the final product [1,2]. Testing for adventitious viruses is therefore an essential quality control step in the manufacture of vaccines and other biological medicines. Non-specific screening for adventitious viruses is partly based on animal tests which have served well for decades, but there are legal and ethical imperatives to replace such tests. Cell culture tests largely solve the ethical issues and are cheaper to perform, and recent efforts to compare sensitivity and specificity with animal tests have been promising [3]. Nevertheless, cell and animal tests are limited by the restricted tropism of some viruses and may not detect non-cytopathic, non-pathogenic or non-haemadsorbing viruses. For example, porcine circovirus (PCV) DNA was detected in two rotavirus vaccines [1,4,5] despite these routine adventitious virus tests showing no evidence of contamination. PCR-based tests offer sensitive and specific detection of their target pathogens, however screening for all potential viruses by PCR is impractical, and non-target viruses would remain undetected.

Deep sequencing (DS, also referred to as massively parallel or high throughput sequencing) offers the potential for identification of extraneous nucleic acid in samples without *a priori* knowledge of the likely contaminant and without the requirement for propagation of the virus. Such methods have already been successfully applied to detection of adventitious agents in vaccines [1], cell lines [6,7], serum [8,9] and bioreactors [10] and multiple laboratory and informatics methods for viral metagenomics have been developed for clinical and other biological specimens [11–17]. There is substantial interest among vaccine manufacturers, contract research organisations, regulators and medicines control laboratories in evaluating the method for routine safety testing, and potentially replacing some or all of the existing *in vitro* and *in vivo* tests. A major challenge to the realisation of this potential is the identification of a robust, sensitive and specific assay design. A wide range of

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Table 1
Virus composition of multiplex reagent 11/242-001.

Group	Family	Envelope	Species/serotype	Genome size (kb)	PCR Ct value	Sample origin
dsDNA	Adenoviridae	No	Adenovirus 2	35.9	29.71	293 cell culture
			Adenovirus 41	34.2	ND	Clinical specimen
	Herpesviridae	Yes	Human herpesvirus 1	151.2	30.59	MRC5 cell culture
			Human herpesvirus 2	154.7	32.48	MRC5 cell culture
			Human herpesvirus 3 (VZV)	124.8	29.02	MeWo cell culture
			Human herpesvirus 4 (EBV)	171.7	31.27	B95-8 cell culture
			Human herpesvirus 5 (CMV)	233.7	28.95	MRC5 cell culture
dsRNA	Reoviridae	No	Rotavirus A	18.5	24.49	Clinical specimen
ssRNA (+)	Astroviridae	No	Astrovirus	6.8	30.53	Clinical specimen
	Caliciviridae	No	Norovirus GI	7.6	ND	Clinical specimen
			Norovirus GII	7.5	ND	Clinical specimen
			Sapovirus C12	7.5	33.37	Clinical specimen
	Coronaviridae	Yes	Coronavirus 229E	27.2	ND	MRC5 cell culture
	Picornaviridae	No	Coxsackievirus B4	7.4	30.72	Hep-2 cell culture
			Rhinovirus A39	7.1	31.16	MRC5 cell culture
			Parechovirus 3	7.2	29.35	LLC-MK2 cell culture
ssRNA (−)	Orthomyxoviridae	Yes	Influenza A virus H1N1	13.2	32.02	Egg passage
			Influenza A virus H3N2	13.6	ND	Egg passage
			Influenza B virus	14.2	ND	Egg passage
	Paramyxoviridae	Yes	Metapneumovirus A	13.3	31.86	LLC-MK2 cell culture
			Parainfluenzavirus 1	15.5	34.43	PRF5 cell culture
			Parainfluenzavirus 2	15.7	33.87	PRF5 cell culture
			Parainfluenzavirus 3	15.4	ND	PRF5 cell culture
			Parainfluenzavirus 4	17.4	31.83	PRF5 cell culture
			Respiratory syncytial virus A2	15.2	34.33	Hep-2 cell culture

ds double-stranded, *ss* single-stranded, *VZV* Varicella Zoster Virus, *EBV* Epstein Barr Virus, *CMV* Cytomegalovirus, *ND* not detectable. Ct values provide a crude estimate of viral genome abundance; quantitative PCR data are not available.

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methods exist for viral metagenomics, many of which are early in their development: multiple options exist for generation of sequencing libraries; several commercial sequencing platforms exist, based on fundamentally different chemistry, with more in development; numerous bioinformatics pipelines are used for sequence classification, both academically and commercially developed; and the databases against which the reads are searched are constantly evolving. Given these parameters, it is important to have suitable reference materials to ensure that different methods generate comparable results. In addition to reagents for comparison of methods and determination of run performance, well-characterised materials of defined virus concentration will be required in order to determine limits of detection for particular viruses or virus types. We describe here a candidate material for qualitative comparison of methods and run performance and its evaluation in an international collaborative study encompassing 15 laboratories. The study highlighted that a broad range of laboratory and informatics techniques are in use, and no consensus exists on the most appropriate combination of methods to achieve maximum sensitivity. We discuss the major challenges for the incorporation of deep sequencing into adventitious agent testing workflows, highlight areas requiring particular attention and describe the requirements of future reference materials to enable validation and comparison of methods.

2. Methods

2.1. Aim and scope

The primary aim of the study was to evaluate the suitability of reagent 11/242-001 as a reference material for deep sequencing-based adventitious virus detection by comparing the results obtained from 15 independent laboratories using a variety of sample preparation, sequencing and informatics methods. Identifying the optimal processing parameters for each step of the process was not feasible given the large number of variables. This study did not aim to assess sensitivity of any particular method, nor the proficiency of the individual laboratories. An outline of this project was presented to the World Health Organisation (WHO) Expert Committee for Biological Standardisation (ECBS) at the 2013 meeting and the committee felt that the project could provide useful information on the value of the reference material and the merits of currently used methods [18].

2.2. Participants

Participants were identified through existing networks of contacts and *via* the Parenteral Drug Association (PDA)/Food and Drug Administration (FDA) Advanced Virus Detection Technologies Interest Group. Participants included vaccine manufacturers, contract research organisations, academic laboratories, regulatory agencies and medicines control laboratories with an interest in virus detection in biological medicines. A full list of participating laboratories is shown in Supplemental Table 1.

2.3. Material

An existing multiplex quantitative polymerase chain reaction (qPCR) run control reagent, 11/242-001, was available for the study. This reagent contains 25 viruses representing a range of common hazard group 2 human viruses (United Kingdom Advisory Committee on Dangerous Pathogens classification) with a variety of genome and envelope types (Table 1).

Individual viruses were propagated in cell culture or by egg passage, and non-cultivable viruses were isolated from clinical specimens. The origin of each virus is described in Table 1. Real-time PCR (RT-PCR) Cycle Threshold (Ct) values were determined for individual virus stocks, and the viruses were then pooled such that the predicted Ct value of each would be approximately 30. Pooled virus was formulated in 10 mM Tris, pH 7.4, supplemented with 2% foetal calf serum. 1 ml of reagent was filled into 2856 2 ml screw-cap Sarstedt vials and frozen at −70 °C. Samples of pooled material were assessed by in-house RT-PCR (see Supplemental Table 2 for PCR conditions) to determine the presence of the 25

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