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A risk-assessment model to rate the occurrence and relevance of adventitious agents in the production of influenza vaccines

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ABSTRACT

Influenza vaccine production has traditionally relied on the use of embryonated chicken eggs for virus isolation and propagation, but recently, cell-culture-derived manufacturing methods have been introduced. During influenza vaccine production, by either conventional or cell culture methods, there is a risk of incidental contamination by adventitious agents. Thus, a risk-assessment model has been developed to qualitatively assess the potential risk of vaccine process contamination by viral pathogens. The model takes into account the basic growth characteristics of each virus, its ability to grow in different cell substrates and resistance to processing steps during vaccine manufacture. The risk-assessment model has been applied to various pathogens to determine potential risk and relevance in different manufacturing scenarios, using different cell substrates for virus propagation, including Madin–Darby canine kidney (MDCK) cells. Avian viruses, introduced via use of embryonated eggs for virus isolation, were found to present the greatest risk, irrespective of the substrate used for influenza virus propagation. The use of MDCK cells to propagate vaccine virus from egg-isolated influenza virus strains does not introduce a new or greater adventitious virus risk, compared with egg-based vaccine production. Indeed, the adventitious virus risk is potentially reduced as fewer viruses are able to grow in MDCK cells.

1. Introduction

The annual assessment of circulating influenza virus strains, and resulting frequent updates to the composition of influenza vaccines, is essential to afford protection against evolving influenza viruses. Due to the timing of communication of composition to manufacturers, influenza vaccines are produced according to a rigid timescale, to ensure availability of the product before the onset of the influenza season. Any evidence of contamination observed during the compressed period of vaccine manufacture would be critical. Contaminated raw materials, process intermediates or influenza vaccine would have to be destroyed, irrespective of the form of the contaminant, unless the contaminating agent could be identified and it could be convincingly demonstrated that the agent could be effectively inactivated and removed. In a worst-case scenario, all production material would have to be disposed of, severely limiting influenza vaccine stocks for the upcoming influenza season.

Conventional inactivated influenza vaccines are produced using embryonated chicken eggs for influenza virus isolation, seed virus preparation and influenza virus propagation [1]. Alternative influenza vaccine production technologies currently in development include the use of mammalian cell lines for virus propagation [2]. Cell-culture-based manufacturing systems could offer more production control, allow aseptic handling during virus production, and facilitate purification of the vaccine product [3–5].

The influenza virus strains used in vaccine manufacture originate from human nasal or throat swabs and may be contaminated by other viruses. Passaging of influenza virus in embryonated eggs also carries the risk of the introduction of avian viruses. The influenza vaccine production timescale only permits limited virus exclusion testing of the virus isolates or seed virus preparations (e.g. tests for avian retroviruses).

To date, there are no published data on the presence of adventitious agents in the influenza vaccine production process, nor in the final vaccine product. However, there have been a number of published reports on the presence of retroviral contaminants and reverse transcriptase activity in other vaccines produced from embryonated chicken eggs or primary chicken embryonic fibroblasts [6–8]. Furthermore, simian virus 40 exposure in subjects receiving polio vaccine produced using poliovirus isolated in monkey kidney cells has been well documented [9].

The viral safety of conventional, inactivated influenza vaccines largely relies on the perceived limited growth of human viruses in embryonated eggs (virus filter effect) and on efficient virus inactivation during purification steps. However, little is known about the

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Table 1
Assignment of scores for evaluated characteristics

Characteristic	Description	Score	Rationale
Growth speed and titres	Fast growth and high titres	2	High titres of agent achieved in ≤3-4 days under conditions used for influenza virus propagation. Risk increased.
	Slow growth OR low titres	1	Neutral intermediate score. Risk level maintained.
	Slow growth AND low titres	0.5	Agent deselected by influenza growth conditions. Moderate risk reduction.
Cell-free transmission	Effectively released	1	Agent contained in fraction used for influenza virus passage. Risk level maintained.
	Cell-associated	0.5	Agent deselected as influenza virus is passaged without cell. Moderate risk reduction.
Growth in cell substrate	Unrestricted growth	2	Adventitious agent grows to high titres in cell substrate. Risk increased.
	Limited growth	1	Neutral intermediate score. Risk level maintained.
	Growth unconfirmed or unlikely	0.5	Moderate risk reduction.
	Unable to grow	0.1	High risk reduction.
	Not present	0	Scoring not applicable.
Stability	Very stable	1.5	Assume agent concentration exceeds agent degradation. Risk increased.
	Stable	1	Assume agent concentration is offset by agent degradation. Risk level maintained.
	Unstable	0.5	Assume agent degradation exceeds agent concentration and so infectivity is reduced. Moderate risk reduction.
Resistance to inactivation	High	0.5	Moderate inactivation expected. Moderate risk reduction.
	Intermediate	0.3	Incomplete inactivation expected. Intermediate risk reduction.
	Low	0.1	Complete inactivation expected. High risk reduction.
Resistance to splitting	Non-enveloped	1	Agent resistant to splitting. Risk level maintained.
	Enveloped	0.5	Agent susceptible to splitting. Moderate risk reduction.
Human pathogenicity	High	10	Agent highly pathogenic in humans. Risk increased.
	Moderate	5	Agent moderately pathogenic in humans. Risk increased.
	None/none known	1	Agent not (or not known to be) pathogenic in humans. Contamination risk level maintained.

virus filter effect of egg passages. Furthermore, published data on the capacity of the vaccine processes in place to inactivate adventitious agents are not available for systematic review, and it is often assumed that the inactivation process completely inactivates likely viral contaminants. Thus, the viral safety of conventional influenza vaccines is primarily based upon historical safety records rather than specific data and facts.

A novel risk-assessment model has been developed to qualitatively assess the risk of contamination of inactivated split and subunit influenza vaccines by a range of viruses and other agents that are not covered by bacterial sterility tests. To ensure broad applicability of the assessment, only common elements of the influenza vaccine production process were taken into account. In addition, to facilitate comparison of different viruses (despite the limited availability of data), relevant characteristics were rated in very basic, non-quantitative or semi-quantitative terms. The risk-assessment model uses a simple scoring approach (e.g. high, intermediate, or low) based on the characteristics of individual viruses, and an overall process risk-score is then calculated for each potential contaminant. The risk-assessment model can be used to identify potential contamination risks in influenza vaccine manufacture. It can also be used to compare and rate the relative risk associated with any of the viruses. The risk-assessment model does not take into account specific manufacturing processes and so can also serve as a useful tool to compare the relevance of any potential contaminant to different vaccine manufacturing processes using alternative cell substrates for the isolation and propagation of influenza virus. Risk control and risk reduction measures for different processes can then be developed, based on this initial assessment.

In this paper, the risk-assessment model has been used to determine the relative risk of a range of potential contaminating viruses in a number of influenza vaccine manufacturing scenarios. The primary objective was to compare the potential contamination risk of a proprietary Madin–Darby canine kidney (MDCK) 33016 cell-culture-derived influenza vaccine (OPTAFLU®, Novartis Vaccines) with that of conventional influenza vaccines. Risk has also been calculated for other cell substrates, or combinations thereof, including Vero cells, because of their potential application in the production of reassortant strains using reverse genetics [10]. The risk-assessment model has been applied to 23 viruses, and *Mycoplasma* and *Chlamydia* species, which could theoretically be introduced into the influenza vaccine manufacturing process either directly or through the influenza virus isolates. It is not designed, or intended, to replace a process- or product-specific assessment and does not allow a specific conclusion to be reached on the safety of any particular vaccine product. The purpose of the risk assessment is to provide an initial overview on process contamination risks, which can be used to prioritise measures taken to improve process robustness and product safety.

2. Methods

2.1. Risk-assessment model

The risk assessment assigns scores to specific agents on a number of parameters based on their characteristics, including growth properties, stability and susceptibility to inactivation, as discussed in further detail below. The characteristics evaluated, and the scores assigned to each characteristic, are summarised in Table 1, and described in more detail below. An overall process risk score is then calculated for each specific agent using a defined algorithm (Fig. 1). The simple algorithm is based on multiplication of the scores assigned to each characteristic and so is designed to establish whether a risk level is maintained, reduced or increased at points in the vaccine manufacturing process. For each characteristic evaluated, a score of <1 denotes a reduced risk, a score of >1 denotes an increase in risk and a score of 1 indicates that the risk level is

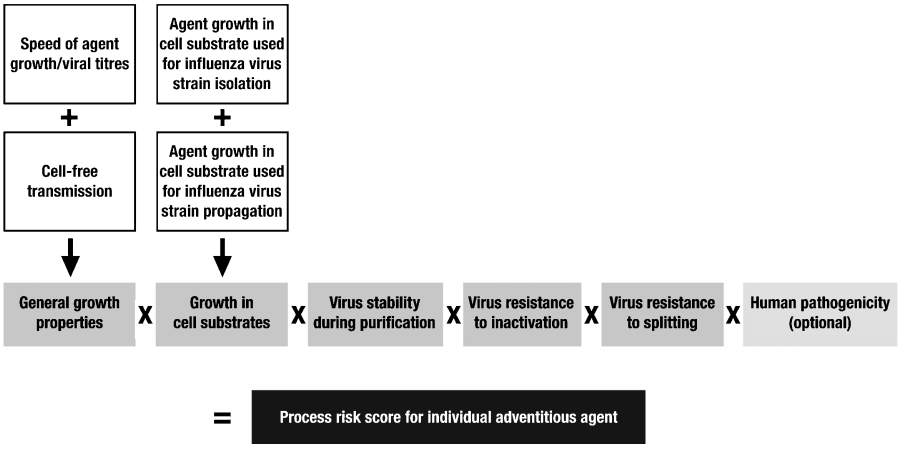

Fig. 1. Calculation of overall process risk score for individual adventitious agents.

maintained. A score of 0 is given where assignment of a score is not appropriate (for example, an avian virus in a solely cell culture manufacturing scenario), and only where it forms part of a subcategory, where two scores are added rather than multiplied, so that calculated final scores will never be 0.

The risk assessment assigns scores based on simple qualitative definitions, due to the limited availability of quantitative data, although where possible, quantitative terms (e.g. virus growth titres) were incorporated. Accepting a certain degree of imprecision, the scores derived from the risk assessment provide a scale on which different virus characteristics can be compared. For simple YES or NO parameters, only two scores were given: 1 for maintained risk or 0.5 for reduced risk. Where greater differentiation could be identified, scores were split further (e.g. for virus growth, score 2 for high virus titre/increased risk; score 1 for maintained virus titre/maintained risk; score 0.5 for moderately lower virus titre/decreased risk). As shown in Fig. 1, the two scores attributed to the general growth properties category, and the two scores attributed to the growth in cell substrate category, are added before the combined score for each category is fed into the algorithm. The subordinate categories are not independent of each other, as a low score in one subcategory will not necessarily result in a reduction in the total risk for that whole category, and so multiplication within the categories would inadequately reduce high risks. Addition of the subcategory scores maintains a higher risk score and adds weight to the risk conferred by viral growth. This is considered appropriate since viral growth is one of the most relevant risk factors in vaccine manufacture.

2.2. General growth properties

During influenza vaccine manufacture, inoculation of the cell substrate with the influenza strain is carried out at a high dilution to avoid growth inhibition by defective interfering influenza virus. Furthermore, as maximum influenza virus titres are usually achieved in 2–3 days, only contaminating viruses that grow rapidly and are effectively released from the cell substrate are expected to yield significant virus titres. Therefore, scores were attributed to specific agents based on their speed of growth (maximum score of 2) and cell-free transmission (maximum score of 1) and added together to give a single score for their general growth properties. Scores were based on data on growth properties in the published literature [11–14] and from our own studies.

Viruses that can achieve high titres in short periods of time (e.g. peak titre >6 $\log_{10}$ tissue culture infectious dose [$TCID_{50}$]/ml in ≤3–4 days) were assigned a score of 2. Viruses with either slow growth or low titres were assigned a score of 1. Viruses with both slow growth and low titres (e.g. peak titre <6 $\log_{10}$ $TCID_{50}$/ml in >4 days) were assigned a score of 0.5.

For cell-free transmission, viruses that are effectively released from the cell substrate were given a score of 1 and those that are mainly transmitted from cell to cell were assigned a score of 0.5. The minimum combined score for any given agent was 1, recognising that this score cannot lower calculated risk.

2.3. Virus growth in different cell substrates

Different cell substrates present different risks for each contaminating agent. Therefore, scores were attributed to specific agents based on their ability to grow in the cell substrates used for influenza virus isolation and propagation (eggs, MDCK cells and Vero cells). Where possible, scores were attributed based on data derived from the published literature [11–27].[1]

For standard adherent MDCK and Vero cells, culture in serum-containing medium or using serum-derived supplements was assumed, as in most scenarios these cells were used during isolation and early passaging of influenza virus. For standard MDCK cells, growth data were entirely derived from published data. For vaccine manufacturing scenarios using MDCK 33016 suspension cells, and thus serum-free growth conditions, viral growth was based on our own study results and so was distinguished from viral growth in standard MDCK cells, i.e. those commonly acquired from the American Type Culture Collection. Suspension cell cultures were inoculated to contain 10^4 $TCID_{50}$/ml of each virus to be tested, and virus titres were monitored for at least 14 days. For all substrate types, certain simplifications were made for virus types from the same family; for example, parainfluenza III growth properties were taken as representative of parainfluenza I, II and IV, even though the latter species exhibit more restricted replication compared with parainfluenza III.

[1] To confirm the absence of evidence for the growth of specific viruses in a cell substrate, a systematic literature search was carried out using the NLM Gateway of the US National Library of Medicines (http://gateway.nlm.nih.gov/gw/Cmd). No publication date limit and no language selection were applied. Consumer Health was excluded as a category. In an NLM Gateway search, the search terms are automatically combined (AND as default Boolean operator). Unqualified terms typed into the query box are automatically mapped according to MeSH terms and searched as such, while the original term entered is also searched as text words. Therefore, simple terms with a broader scope and no Boolean operators were used as preferred search entries. The standard search scheme included the virus name and the virus family and subgroup, in combination with the cell type.

Table 2
Individual scores for each characteristic evaluated and overall process scores for each pathogen according to a conventional egg-based, vaccine-manufacturing scenario (egg > egg)

Virus group	Virus family	Type/species	Individual score for each characteristic evaluated							Process score	Rank
			General growth properties		Viral growth in cell substrate		Process resistance				
			Growth speed and titres	Cell-free transmission	Isolation (eggs)	Propagation (eggs)	Stability	Inactivation	Splitting		
-RNA	Paramyxoviridae	Pneumovirus/hRSV	0.5	0.5	0.1	0.1	0.5	0.1	0.5	0.01	24
		Metapneumovirus	0.5	1	0.5	0.5	0.5	0.1	0.5	0.04	22
		Parainfluenzavirus	2	1	2	2	0.5	0.1	0.5	0.30	11
		Mumps virus	1	1	1	1	0.5	0.1	0.5	0.10	18
		Measles virus	1	1	0.5	0.5	0.5	0.1	0.5	0.05	19
+RNA	Coronaviridae	HCoV, SARS-CoV	1	1	0.5	0.5	0.5	0.1	0.5	0.05	19
	Togaviridae	Rubella virus	1	1	0.5	0.5	0.5	0.1	0.5	0.05	19
	Picornaviridae	Human enterovirus	2	1	0.5	0.5	1	0.3	1	0.90	9
		Rhinovirus	1	1	0.5	0.5	1	0.1	1	0.20	14
ss RNA(RT)	Retroviridae	Avian retrovirus	1	0.5	2	2	0.5	0.3	0.5	0.45	10
		Human retrovirus	1	0.5	0.1	0.1	0.5	0.3	0.5	0.02	23
ds RNA	Reoviridae	Mammalian reovirus	2	1	1	1	1.5	0.5	1	4.50	4
		Avian reovirus	2	1	2	2	1.5	0.5	1	9.00	1
	Birnaviridae	Avian birnavirus	2	1	2	2	1.5	0.5	1	9.00	1
ss DNA	Parvoviridae	MVM	1	1	0.5	0.5	1.5	0.1	1	0.30	11
	Circoviridae	Avian circovirus	1	1	1	1	1.5	0.3	1	1.80	5
		Porcine circovirus	0.5	0.5	0	0	1.5	0.3	1	0.00	24
ds DNA	Herpesviridae	Herpes simplex virus	2	1	2	2	0.5	0.5	0.5	1.50	6
		Varicella zoster virus	1	1	0.5	0.5	0.5	0.5	0.5	0.25	13
		EBV, CMV, HHV-6-8	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.13	17
	Adenoviridae	Human adenovirus	2	1	0.5	0.5	1	0.5	1	1.50	6
	Polyomaviridae	JC/BK virus, SV40	1	1	0.5	0.5	1.5	0.5	1	1.50	6
		Avian polyomavirus	2	1	2	2	1.5	0.5	1	9.00	1
Other agents	–	*Mycoplasma* spp.	1	0.5	2	2	0.5	0.1	0.5	0.15	15
	–	*Chlamydia* spp.	1	0.5	2	2	0.5	0.1	0.5	0.15	15

CMV, cytomegalovirus; ds, double-stranded; EBV, Epstein–Barr virus; HCoV, human coronavirus; HHV, human herpes virus; hRSV, human respiratory syncytial virus; MVM, minute virus of mice; SARS-CoV, severe acute respiratory syndrome coronavirus; ss, single-stranded; SV40, simian virus 40.

Where contaminating virus growth is unrestricted in the cell substrate (e.g. peak titre >6 $\log_{10}$ $TCID_{50}$/ml or >3 $\log_{10}$ above the inoculum titre), a score of 2 was assigned. Limited growth of a contaminating virus (e.g. peak titre <6 $\log_{10}$ $TCID_{50}$/ml or <3 $\log_{10}$ above the inoculum titre), or those viruses requiring adaptation to grow in the cell substrate, were assigned a score of 1. If virus growth could not be confirmed, or if growth of a particular virus was deemed unlikely, a score of 0.5 was given. Where the inability of a particular contaminating agent to grow in a specific cell substrate has been confirmed experimentally, a score of 0.1 was given. A score of 0 was assigned where the specific agent being assessed is not present in the cell substrate, for example, an avian virus in a solely cell culture manufacturing scenario.

2.4. Virus stability during a purification process

Although the risk-assessment model is not process-specific, viruses were scored according to their capacity to survive a purification process based on their taxonomic characteristics and environmental stability (Table 1). A score of 1.5 was assigned to very stable viruses (e.g. polyomaviruses, parvoviruses, reoviruses) assuming that the virus concentration during the process might be greater than the total virus degradation. A score of 1 was assigned to stable viruses (e.g. rhinoviruses, enteroviruses, adenoviruses). Where greater losses of virus were expected, for example, the unstable viruses, a score of 0.5 was assigned.

2.5. Virus resistance to inactivation

As viruses have varying degrees of susceptibility to inactivation processes, scores were assigned to specific agents based on their taxonomic characteristics and, therefore, their likely resistance to inactivation. Scores were attributed based on data from our own studies evaluating the inactivation of a range of viruses, covering all taxonomic properties, using beta-propiolactone and formaldehyde, essentially the only inactivating agents used in the manufacture of vaccines for human use. Since the inactivation process will inactivate any virus to a certain degree, all scores attributed were below 1 (Table 1). Viruses that exhibit some resistance to inactivation by such agents (e.g. virus titre reduced by <3 $\log_{10}$ steps by commonly applied inactivation process conditions) were assigned a score of 0.5. Viruses that show a marked but not complete reduction (e.g. a 4–5 $\log_{10}$ reduction) were assigned a score of 0.3. Finally, for those viruses that are inactivated to below the level of detection of the assay (e.g. reduced by $\geq$6 $\log_{10}$ steps), a score of 0.1 was assigned.

2.6. Virus resistance to splitting

All split and subunit influenza vaccines undergo a detergent treatment step to disrupt virions or to release influenza virus surface and membrane antigens. Non-enveloped viruses are resistant to such treatment, so were assigned a score of 1; enveloped viruses are susceptible to detergent disruption and were assigned a score of 0.5 (Table 1).

2.7. Human pathogenicity

A separate assessment was performed to rate and rank the relevance of each virus in terms of pathogenicity in humans. Scores were attributed based on the degree of known pathogenicity: viruses known to be highly pathogenic in humans, including human

Table 3
Individual scores for each characteristic evaluated and overall process scores for each pathogen according to influenza virus isolation in eggs and influenza virus propagation in MDCK 33016 cells (egg > MDCK manufacturing scenario)

Virus group	Virus family	Type/species	Individual score for each characteristic evaluated							Process score	Rank
			General growth properties		Viral growth in cell substrate		Process resistance				
			Growth speed and titres	Cell-free transmission	Isolation (eggs)	Propagation (MDCK 33016)	Stability	Inactivation	Splitting		
-RNA	Paramyxoviridae	Pneumovirus/hRSV	0.5	0.5	0.1	0.1	0.5	0.1	0.5	0.01	25
		Metapneumovirus	0.5	1	0.5	0.5	0.5	0.1	0.5	0.04	22
		Parainfluenzavirus	2	1	2	2	0.5	0.1	0.5	0.30	10
		Mumps virus	1	1	1	1	0.5	0.1	0.5	0.10	15
		Measles virus	1	1	0.5	1	0.5	0.1	0.5	0.08	18
+RNA	Coronaviridae	HCoV, SARS-CoV	1	1	0.5	0.1	0.5	0.1	0.5	0.03	23
	Togaviridae	Rubella virus	1	1	0.5	0.5	0.5	0.1	0.5	0.05	20
	Picornaviridae	Human enterovirus	2	1	0.5	0.1	1	0.3	1	0.54	9
		Rhinovirus	1	1	0.5	0.1	1	0.1	1	0.12	14
ss RNA(RT)	Retroviridae	Avian retrovirus	1	0.5	2	0.1	0.5	0.3	0.5	0.24	12
		Human retrovirus	1	0.5	0.1	0.1	0.5	0.3	0.5	0.02	24
ds RNA	Reoviridae	Mammalian reovirus	2	1	1	1	1.5	0.5	1	4.50	4
		Avian reovirus	2	1	2	1	1.5	0.5	1	6.75	1
	Birnaviridae	Avian birnavirus	2	1	2	0.1	1.5	0.5	1	4.73	2
ss DNA	Parvoviridae	MVM	1	1	0.5	0.1	1.5	0.1	1	0.18	13
	Circoviridae	Avian circovirus	1	1	1	0.5	1.5	0.3	1	1.35	6
		Porcine circovirus	0.5	0.5	0	0.1	1.5	0.3	1	0.05	21
ds DNA	Herpesviridae	Herpes simplex virus	2	1	2	2	0.5	0.5	0.5	1.50	5
		Varicella zoster virus	1	1	0.5	0.5	0.5	0.5	0.5	0.25	11
		EBV, CMV, HHV-6-8	0.5	0.5	0.5	0.1	0.5	0.5	0.5	0.08	19
	Adenoviridae	Human adenovirus	2	1	0.5	0.1	1	0.5	1	0.90	7
	Polyomaviridae	JC/BK virus, SV40	1	1	0.5	0.1	1.5	0.5	1	0.90	7
		Avian polyomavirus	2	1	2	0.1	1.5	0.5	1	4.73	2
Other agents	–	*Mycoplasma* spp.	1	0.5	2	0.1	0.5	0.1	0.5	0.08	16
	–	*Chlamydia* spp.	1	0.5	2	0.1	0.5	0.1	0.5	0.08	16

CMV, cytomegalovirus; ds, double-stranded; EBV, Epstein–Barr virus; HCoV, human coronavirus; HHV, human herpes virus; hRSV, human respiratory syncytial virus; MDCK, Madin–Darby canine kidney; MVM, minute virus of mice; SARS-CoV, severe acute respiratory syndrome coronavirus; ss, single-stranded; SV40, simian virus 40.

retroviruses and severe acute respiratory syndrome coronavirus (SARS-CoV), scored 10; viruses known to be moderately pathogenic scored 5 and viruses with no (or no known) pathogenicity, including avian and other animal viruses, scored 1. Viruses could not score below 1, recognising that the pathogenicity factor cannot reduce the contamination risk.

3. Results

The risk-assessment model has been applied to various virus isolation/virus propagation scenarios that can be used in influenza vaccine production: (1) isolation and propagation in eggs (conventional influenza vaccine manufacture); (2) isolation of the influenza virus in embryonated eggs and propagation in MDCK 33016 cells (the OPTAFLU® manufacturing process); (3) isolation in eggs and propagation in Vero cells; (4) isolation in eggs, further passages in Vero cells and propagation in MDCK 33016 cells; (5) isolation in Vero cells and propagation in MDCK 33016 cells; (6) isolation in standard MDCK cells and propagation in MDCK 33016 cells. The individual scores attributed for general growth properties and stability, inactivation and splitting are the same for each manufacturing scenario evaluated; only scores for viral growth in cell substrate differ between scenarios.

The full scoring system for the risk-assessment calculation for the 23 viruses, *Mycoplasma* species and *Chlamydia* species, with regard to a conventional egg-based vaccine manufacturing scenario, is presented in Table 2. The table shows the individual scores assigned for each evaluated characteristic, and the calculated process score and associated rank with regard to each pathogen. The non-enveloped avian birnavirus, avian polyomavirus and avian reovirus were attributed the highest overall process score of 9.00, due to their high growth potential in eggs. Mammalian reovirus also scored highly, ranking fourth after the avian viruses. The calculated process scores for most enveloped viruses, including the coronaviridae and the paramyxoviridae, were generally low, although herpes simplex virus (HSV) scored quite highly in the assessment (1.50).

Table 3 presents the full scoring system for the same range of viruses when eggs are used for influenza virus isolation and MDCK 33016 suspension cells for influenza virus propagation. Again, the non-enveloped avian birnavirus, avian polyomavirus and avian reovirus were attributed the highest overall process scores, ranging from 4.73 to 6.75. Like many other cell substrates, MDCK 33016 cells support growth of mammalian reovirus, as demonstrated in our own studies, and so mammalian reovirus also scored highly. Conversely, our own studies have shown that adenoviruses and polyomaviruses do not grow in MDCK 33016 cells, therefore, these viruses were associated with a lower risk when MDCK 33016 cells were used for influenza virus propagation, compared with a conventional egg-based manufacturing process.

Table 4 shows the calculated overall process scores for all six manufacturing scenarios evaluated, together with the average risk scores and rank for each pathogen across the different scenarios. With a few exceptions, similar process scores were obtained for each pathogen, regardless of the manufacturing scenario, with high scores attributed to avian reovirus, avian birnavirus and avian polyomavirus. Mammalian reovirus also achieved high scores against each manufacturing scenario. Adenoviruses and JC/BK polyomaviruses achieved 2- to 4-fold higher scores if Vero cells are to

Table 4
Process scores for manufacturing scenarios using different cell substrates for virus isolation and manufacturing

Virus group	Virus family	Type/species	Process score for each manufacturing scenario[a]						Average score for virus	Rank
			Egg > egg	Egg > MDCK 33016	Egg > Vero	Egg > Vero > MDCK 33016	Vero > MDCK 33016	Normal MDCK > MDCK 33016		
-RNA	Paramyxoviridae	Pneumoviruses/hRSV	0.0	0.0	0.1	0.1	0.1	0.0	0.03	24
		Metapneumovirus	0.0	0.0	0.1	0.1	0.1	0.0	0.05	23
		Parainfluenzavirus	0.3	0.3	0.3	0.5	0.3	0.3	0.33	11
		Mumps virus	0.1	0.1	0.2	0.2	0.2	0.1	0.13	16
		Measles virus	0.1	0.1	0.1	0.1	0.1	0.1	0.09	19
+RNA	Coronaviridae	HCoV, SARS-CoV	0.1	0.0	0.1	0.1	0.1	0.0	0.08	21
	Togaviridae	Rubella virus	0.1	0.1	0.1	0.2	0.1	0.1	0.09	18
	Picornaviridae	Human enteroviruses	0.9	0.5	2.3	2.3	1.9	1.0	1.49	9
		Rhinovirus	0.2	0.1	0.5	0.5	0.4	0.1	0.31	12
ss RNA (RT)	Retroviridae	Avian retroviruses	0.5	0.2	0.2	0.2	–	–	0.29	13
		Human retroviruses	0.0	0.0	0.0	0.0	0.0	0.0	0.02	25
ds RNA	Reoviridae	Mammalian reovirus	4.5	4.5	6.8	9.0	6.8	6.8	6.38	3
		Avian reovirus	9.0	6.8	9.0	11.3	–	–	9.00	1
	Birnaviridae	Avian birnavirus	9.0	4.7	9.0	9.2	–	–	7.99	2
ss DNA	Parvoviridae	MVM	0.3	0.2	0.3	0.3	0.2	0.1	0.23	15
	Circoviridae	Avian circovirus	1.8	1.4	1.4	1.8	–	–	1.58	8
		Porcine circovirus	0.0	0.0	0.5	0.5	0.5	0.1	0.26	14
ds DNA	Herpesviridae	Herpes simplex virus	1.5	1.5	1.5	2.3	1.5	1.5	1.63	7
		Varicella zoster virus	0.3	0.3	0.6	0.8	0.6	0.3	0.46	10
		EBV, CMV, HHV-6-8	0.1	0.1	0.1	0.1	0.1	0.1	0.10	17
	Adenoviridae	Human adenovirus	1.5	0.9	3.8	3.9	3.2	1.7	2.48	5
	Polyomaviridae	JC/BK virus, SV40	1.5	0.9	3.8	3.9	3.2	0.9	2.35	6
		Avian polyomavirus	9.0	4.7	5.6	5.9	–	–	6.30	4
Other agents	–	*Mycoplasma* spp.	0.2	0.1	0.1	0.1	0.0	0.0	0.08	22
	–	*Chlamydia* spp.	0.2	0.1	0.1	0.1	0.0	0.0	0.08	20
Summed process scores for each scenario			41	28	46	53	19	13	–	–

CMV, cytomegalovirus; ds, double-stranded; EBV, Epstein–Barr virus; HCoV, human coronavirus; HHV, human herpes virus; hRSV, human respiratory syncytial virus; MDCK, Madin–Darby canine kidney; MVM, minute virus of mice; SARS-CoV, severe acute respiratory syndrome coronavirus; ss, single-stranded; SV40, simian virus 40.

[a] Process scores have been rounded to one decimal place.

be used for influenza virus propagation, compared with scenarios not using Vero cells (Table 4). Similarly, scores for human picornaviridae increased when Vero cells were involved. *Mycoplasma* species and *Chlamydia* species achieved low scores in all assessments, regardless of the cell substrate used for influenza virus isolation and propagation.

Table 4 also shows the summed process score for each manufacturing scenario, which gives an indication of the relative risk of contamination associated with each scenario. A conventional egg-based manufacturing process (egg > egg) scenario produced a summed score of 41, compared with 28 for virus isolation in eggs and production in MDCK 33016 cells (eggs > MDCK 33016 cells). None of the individual scores in the egg > MDCK 33016 scenario was higher than those in the egg > egg scenario. Furthermore, an MDCK > MDCK 33016 scenario, where MDCK cells are also used for virus isolation, produced an even lower summed score of 13; higher summed scores were obtained when Vero cells were involved in the process.

An additional assessment has been carried out, taking into consideration the pathogenic potential of each virus in humans in the calculation of the overall process score. Table 5 shows the final process scores for each virus with the human pathogenicity score included. As would be expected, human viruses scored highly, with mammalian reovirus, human adenovirus and JC/BK polyomavirus achieving the highest scores. Herpes simplex virus and human enterovirus also scored highly. Despite their lack of human pathogenicity, relatively high scores were attributed to avian viruses, including reovirus, birnavirus and polyomavirus, if eggs were involved in the manufacturing process. Inclusion of a pathogenicity factor in the process scores did not affect the ranking of each manufacturing process in terms of summed process scores; scores were lower when eggs were replaced with MDCK 33016 cells and inclusion of Vero cells produced a higher summed score.

4. Discussion

Using the risk-assessment model, we have shown that avian viruses, which may be introduced via the use of embryonated eggs, represent the greatest risk for adventitious contamination of an inactivated subunit influenza vaccine. Interestingly, less well-known avian viruses, such as reovirus, birnavirus and polyomavirus, are the greatest threat, whereas avian retroviruses appear to be less relevant. As would be expected, avian viruses generally scored high when eggs were used for influenza virus propagation as well as influenza virus isolation.

Although most avian viruses are apathogenic in humans [28], and so may be less relevant to the final influenza vaccine than human pathogens, any kind of contamination during influenza vaccine manufacture could disrupt vaccine production and so hinder the timely distribution of influenza vaccine. As would be expected, if embryonated eggs are replaced by MDCK cell cultures for the isolation of influenza strains, the risk of avian virus entering the vaccine manufacturing process is eliminated.

Of the human viruses analysed, most well-known pathogens achieved low scores, while mammalian reovirus proved to carry the highest risk in this assessment. Reoviruses are common in all species and tend to grow in almost any cell type. Our own studies have shown reoviruses to be very resistant to chemical inactivation

Table 5
Process scores for each manufacturing scenario, including human pathogenicity score

Virus group	Virus family	Type/species	Process score for each manufacturing scenario[a]						Average score for virus	Rank
			Egg > egg	Egg > MDCK 33016	Egg > Vero	Egg > Vero > MDCK 33016	Vero > MDCK 33016	Normal MDCK > MDCK 33016		
-RNA	Paramyxoviridae	Pneumoviruses/hRSV	0.0	0.0	0.3	0.3	0.3	0.1	0.15	25
		Metapneumovirus	0.2	0.2	0.3	0.4	0.3	0.2	0.25	22
		Parainfluenzavirus	1.5	1.5	1.5	2.3	1.5	1.5	1.63	10
		Mumps virus	0.5	0.5	0.8	1.0	0.8	0.5	0.67	14
		Measles virus	0.3	0.4	0.4	0.6	0.5	0.5	0.44	17
+RNA	Coronaviridae	HCoV, SARS-CoV	0.5	0.3	1.3	1.3	1.1	0.3	0.78	13
	Togaviridae	Rubella virus	0.3	0.3	0.6	0.8	0.6	0.3	0.46	16
	Picornaviridae	Human enteroviruses	4.5	2.7	11.3	11.7	9.5	5.0	7.43	7
		Rhinovirus	1.0	0.6	2.5	2.6	2.1	0.6	1.57	12
ss RNA (RT)	Retroviridae	Avian retroviruses	0.5	0.2	0.2	0.2	–	–	0.29	20
		Human retroviruses	0.2	0.2	0.2	0.3	0.2	0.2	0.24	23
ds RNA	Reoviridae	Mammalian reovirus	22.5	22.5	33.8	45.0	33.8	33.8	31.88	1
		Avian reovirus	9.0	6.8	9.0	11.3	–	–	9.00	4
	Birnaviridae	Avian birnavirus	9.0	4.7	9.0	9.2	–	–	7.99	6
ss DNA	Parvoviridae	MVM	0.3	0.2	0.3	0.3	0.2	0.1	0.23	24
	Circoviridae	Avian circovirus	1.8	1.4	1.4	1.8	–	–	1.58	11
		Porcine circovirus	0.0	0.0	0.5	0.5	0.5	0.1	0.26	21
ds DNA	Herpesviridae	Herpes simplex virus	7.5	7.5	7.5	11.3	7.5	7.5	8.13	5
		Varicella zoster virus	1.3	1.3	3.1	3.8	3.1	1.3	2.29	9
		EBV, CMV, HHV-6-8	0.6	0.4	0.6	0.7	0.4	0.4	0.51	15
	Adenoviridae	Human adenovirus	7.5	4.5	18.8	19.5	15.8	8.3	12.38	2
	Polyomaviridae	JC/BK virus, SV40	7.5	4.5	18.8	19.5	15.8	4.5	11.75	3
		Avian polyomavirus	9.0	4.7	5.6	5.9	–	–	6.30	8
Other agents	–	*Mycoplasma* spp.	0.8	0.4	0.5	0.5	0.1	0.1	0.39	19
	–	*Chlamydia* spp.	0.8	0.4	0.6	0.6	0.2	0.0	0.42	18
Summed process scores for each scenario			87	66	129	151	94	65	–	–

CMV, cytomegalovirus; ds, double-stranded; EBV, Epstein–Barr virus; HCoV, human coronavirus; HHV, human herpes virus; hRSV, human respiratory syncytial virus; MDCK, Madin–Darby canine kidney; MVM, minute virus of mice; SARS-CoV, severe acute respiratory syndrome coronavirus; ss, single-stranded; SV40, simian virus 40.
[a] Process scores have been rounded to one decimal place.

and to be more likely to survive downstream purification steps due to their stable, double-stranded nucleic acid material and their capsid structure, compared with non-enveloped viruses. Adenoviruses, polyomaviruses and enteroviruses were also linked with rather higher risks than other pathogens, particularly when Vero cells were used at any stage. Enveloped viruses are generally susceptible to chemical inactivation methods and to the detergent treatment used in the downstream processing during influenza vaccine manufacture, and so most enveloped viruses achieved low scores, with the exception of HSV. From our own studies and the published literature, it was found that HSV grows rapidly in all the cell substrates evaluated in this assessment [12,15,16,22].

Using this risk-assessment model, we have shown that using MDCK 33016 cells for influenza virus propagation from an egg-derived influenza isolate (egg > MDCK 33016) potentially reduces the chance of viral contamination compared with conventional egg-based influenza vaccine manufacture (egg > egg), as fewer viruses are able to grow in the MDCK 33016 cell substrate. Using MDCK cells for virus propagation did not increase the relative risk posed by any human pathogen compared with conventional egg-based vaccine manufacture.

The manufacturing process involving both isolation and production of influenza virus in MDCK cells was associated with the lowest risk of viral contamination, as the threat of avian contaminants from eggs was removed. Therefore, this qualitative assessment supports the suitability of MDCK cells for both influenza virus isolation and production. This is an important finding as MDCK cells have been used for influenza virus isolation, but such isolates have not yet been used for vaccine manufacture due to the unknown risk of contamination by extraneous viruses. Currently, only egg-derived isolates are used for influenza vaccine manufacture. Furthermore, use of MDCK cells for both influenza virus isolation and production may provide vaccines that are a closer antigenic match to influenza strains circulating in the human population than vaccines in which virus strains are isolated in eggs [29,30]. A closer antigenic match may result in an improved immunogenic response in vaccine recipients.

When the risk-assessment model was applied to other manufacturing scenarios, the use of Vero cells increased or maintained the overall risk; compared with other scenarios, Vero cells support the growth of a wide range of viruses, including many human pathogens [11–16,18,19,23–25,31]. If Vero cells are used instead of eggs for influenza virus isolation or propagation, contamination with avian viruses is avoided. However, the risk of contamination with other viruses is either maintained or increased, e.g. adenoviruses, polyomaviruses, enteroviruses, rhinoviruses, reovirus, and varicella zoster virus. A combination of eggs for influenza virus isolation and Vero cells for further passaging introduces even higher risks, because of the possibility of contamination with avian viruses. This latter scenario would most likely apply for future applications of Vero cell-based reverse-genetics techniques to produce influenza strains. These plasmid-based techniques include other measures to reduce initial contaminants from the virus strains, but do not exclude contamination originating from the human operator during cell culture.

Incorporating a human pathogenicity factor into the risk assessment did not alter the ranking of viruses in terms of their overall process score. Mammalian reovirus, human adenovirus, JC/BK

polyomavirus and human enterovirus continued to score highly as did several avian viruses, including avian reovirus, birnavirus and polyomavirus.

The results described here apply to inactivated split or subunit influenza vaccines, but by omitting the virus-splitting scoring step, the model could easily be applied to the manufacture of the whole-virus vaccines that are under consideration for pandemic influenza vaccines. To assess live attenuated influenza vaccines, only the scores for general growth properties, growth in cell substrate and stability would be applied.

For more specific applications, the risk-assessment model can also be modified to include virus exclusion tests, for example, polymerase chain reaction or conventional tests in cell cultures or *in vivo*. These can be scored as follows: 0.5 if tests are available and able to detect existing strains, or 1 if there are no suitable tests available. As would be expected, addition of this step would produce major effects on the scores of viruses that grow well in the cell substrates used for virus propagation.

The risk-assessment model presented here can provide an initial overview of adventitious virus risk in the manufacture of influenza vaccines. As the risk assessment is not process-specific, the model offers considerable flexibility and range of application, and should be considered a useful tool to evaluate virus-specific contamination risk during vaccine manufacture. It is important to note that the assessment described here cannot be used to evaluate the viral safety of any specific vaccine in terms of risk to the vaccine recipient. Instead, a process-specific evaluation must be carried out; an example has been described in a separate paper [32].

Specific data relating to process contamination events are rarely published and so the model described here cannot be validated against material data. Instead, this paper should be considered as an attempt to open discussion, and the risk model should be further evaluated using data as and when it becomes available. In particular, more detailed information on the presence and frequency of contaminating viruses in embryonated eggs and in early influenza virus isolates obtained from different cell substrates would be valuable.

Viral safety assessments are normally carried out to determine the consequence of human pathogen contamination on a final vaccine product; however, the primary focus of this risk assessment is on the probability of a contamination event by any virus, irrespective of its pathogenic potential in humans. This is particularly relevant for influenza vaccine manufacture, where discovery of any sort of viral contamination would be critical, and so the same technical counter measures drawn from the risk assessment will apply to both low- and high-pathogenicity viruses. As influenza vaccine manufacturers worldwide are supplied with the same reference virus isolates, and as influenza vaccines are produced according to a rigid timescale, any evidence of non-pathogenic contamination is likely to negatively impact on vaccine manufacture. Not only would this present a commercial risk for vaccine manufacturers, it may also present a public health risk, due to delayed vaccine supply.

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