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A quantitative risk assessment of exposure to adventitious agents in a cell culture-derived subunit influenza vaccine

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ABSTRACT

A risk-assessment model has demonstrated the ability of a new cell culture-based vaccine manufacturing process to reduce the level of any adventitious agent to a million-fold below infectious levels. The cell culture-derived subunit influenza vaccine (OPTAFLU®, Novartis Vaccines and Diagnostics) is produced using Madin–Darby canine kidney (MDCK) cells to propagate seasonal viral strains, as an alternative to embryonated chicken-eggs. As only a limited range of mammalian viruses can grow in MDCK cells, similar to embryonated eggs, MDCK cells can act as an effective filter for a wide range of adventitious agents that might be introduced during vaccine production. However, the introduction of an alternative cell substrate (for example, MDCK cells) into a vaccine manufacturing process requires thorough investigations to assess the potential for adventitious agent risk in the final product, in the unlikely event that contamination should occur.

The risk assessment takes into account the entire manufacturing process, from initial influenza virus isolation, through to blending of the trivalent subunit vaccine and worst-case residual titres for the final vaccine formulation have been calculated for >20 viruses or virus families. Maximum residual titres for all viruses tested were in the range of 10^{-6} to 10^{-16} infectious units per vaccine dose. Thus, the new cell culture-based vaccine manufacturing process can reduce any adventitious agent to a level that is unable to cause infection.

1. Introduction

Currently available inactivated trivalent influenza vaccines are produced from influenza virus propagated in eggs. Relevant influenza virus strains are isolated individually in embryonated chicken-eggs according to World Health Organization (WHO) annual recommendations (current vaccines contain strains A/H3N2, A/H1N1 and B) [1]. The influenza reference strain isolates, or reassortants of the circulating strain and a high-yielding egg-adapted strain, are then distributed to influenza vaccine manufacturers, who use these strains to produce viral seed lots. Using these seed lots, each individual strain is grown separately by inoculating embryonated eggs with the isolated reference strain. After several days' incubation, the virus is harvested and purified, inactivated and combined with the other two purified, inactivated influenza virus strains to produce a trivalent influenza vaccine [2].

Although egg-derived vaccines are efficacious, reducing cases of laboratory-confirmed influenza illness by 70–90% in healthy adults [3], the production of such vaccines is inflexible and limited by the availability of eggs [4]. Furthermore, demand for influenza vaccine is increasing, in part because of the expansion of vaccination recommendations and the potential threat of an influenza pandemic [5,6].

Mammalian cell culture has successfully been employed for the propagation of influenza viruses [7] and subsequently for influenza vaccine manufacture [8]. Utilising mammalian cells instead of embryonated eggs for virus propagation has the potential to overcome technical limitations of current manufacturing practices, including long lead times and the possibility of antigenic mismatch with the circulating influenza strain [4,9,10]. A trivalent subunit influenza vaccine has been produced using Madin–Darby canine kidney (MDCK) cells for virus propagation (OPTAFLU®, Novartis Vaccines and Diagnostics [NVD]) and licensed by the European Medicines Agency.

Before a continuous cell line can be accepted for use in vaccine production, safety assessments must be carried out on the cell line itself and on the vaccine manufacturing process, to confirm the absence of any oncogenic element, for example an oncogenic virus, viral gene or cellular oncogene, as well as to confirm the viral safety of the vaccine.

Assessments include testing for tumourigenic or oncogenic events induced by intact cells, cell lysates and extracted DNA in

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specific *in vivo* models, testing for oncogenic viruses in cell banks, confirming the removal of intact cells during vaccine processing, and the use of specific measures to remove and degrade residual DNA.

Historically, concerns over the viral safety of vaccines have arisen from the potential incidental introduction of adventitious agents into the vaccine manufacturing process [11,12]. In the case of influenza vaccines, adventitious viruses may be present in the original clinical isolate, or may be introduced during passaging of the reference strains in embryonated eggs. Other potential sources of contamination include raw materials, laboratory/process personnel and other external sources. Any biological process carries an adventitious agent risk and, although incidental introduction of adventitious agents could occur during conventional egg-based production of inactivated influenza vaccines, these vaccines have a well-established safety profile [13]. Because of their limited susceptibility to a range of viruses, eggs can act as a filter for a wide range of agents, thereby lowering the adventitious agent risk.

A quantitative risk-assessment model has been developed to determine the product and process-failure risk posed by adventitious agents that might occur during the OPTAFLU® manufacturing process. This process-specific assessment considers all elements of the manufacturing process, and utilises polymerase chain reaction (PCR)-based virus exclusion tests, clearance capability and viral inactivation potential of the vaccine manufacturing process. The assessment starts at the earliest relevant step: the isolation of an influenza virus strain from a human throat sample. It also considers egg passages to isolate influenza virus, MDCK cell passages to prepare viral seeds and PCR detection limits for tested materials, and extends through the entire manufacturing process, including fermentation, purification and blending of the final trivalent vaccine. The risk-assessment model can be applied to a range of potential contaminants and allows an estimation of maximum human infectious doses per vaccine dose to be made. A worst-case scenario is applied to every step of the manufacturing process in order to adequately cover variables such as potential virus titres, virus passage numbers, dilutions or inactivation results that might occur between different virus types of the same virus family.

Here, we report on the application of this risk-assessment model to >20 viruses or virus families that could be theoretically introduced during manufacture of the cell culture-derived vaccine, including human agents such as herpes virus, adenovirus, coronavirus, enterovirus, reovirus, retrovirus, rhinovirus, parainfluenza virus and pneumovirus, in addition to avian retrovirus, reovirus, birnavirus and porcine circovirus. Estimates of maximum residual titres (per millilitre of cell culture-derived vaccine) have been calculated for all viruses/virus families and each has been translated into maximum human infectious units per vaccine dose.

2. Materials and methods

2.1. Cell culture-derived vaccine production process

2.1.1. MDCK 33016 cell line

The MDCK epithelial cell line originated from the kidney of a healthy dog in 1958 [14] and has been adapted by NVD to grow freely suspended in serum-free, chemically defined medium. These cells, termed MDCK 33016, have been used to produce a master cell bank and a working cell bank. Both cell banks have been extensively screened for the presence of adventitious agents, using open-ended detection methods, including conventional *in vivo* and *in vitro* adventitious agent tests and specific tests for human, canine, porcine, bovine, rodent and equine viruses. No evidence of any adventitious agent in either cell bank has been observed. The parent cells of the MDCK cell banks have also been screened extensively for adventitious viral agents with a negative result.

2.1.2. Virus isolation and vaccine reference virus preparation

Reference influenza virus strains, containing those strains recommended annually by the WHO, are supplied by WHO Collaborating Centres. The reference strains are isolated by inoculating embryonated chicken-eggs with human clinical samples, for example, from throat swabs of influenza-infected individuals. Influenza virus growth is then monitored and virus isolates are recovered, analysed according to their antigenicity and genetic phenotype, and selected to match the current circulating influenza strains. As many strains do not grow well in embryonated eggs, some influenza strains undergo reassortment with high-yielding strains that do grow well in eggs [4]. Although the risk of co-isolating adventitious agents alongside influenza virus is reduced by the use of high dilutions of the inoculum and regular haemagglutination testing, no specific measures are in place to purposefully exclude or test for adventitious agents before a specific virus is recommended for vaccine manufacture.

2.1.3. Manufacturing process

With the NVD cell culture-derived vaccine manufacturing process, viral seed stocks for the annual vaccine are prepared by passaging the egg-adapted WHO virus isolates in MDCK 33016 cells. Virus preparations from passages of adequately high titre are frozen and used as working seed virus stocks. Based upon the results of the risk assessments, the working seed-stocks routinely undergo PCR-based testing for selected adventitious agents.

MDCK 33016 cells are cultured in bioreactors at a large-scale working volume of >1000 l. The bioreactor is then inoculated with the influenza seed virus and processing continues until viruses are released into the culture medium. The MDCK cells are removed by centrifugation and the virus-containing supernatant is harvested. At this point, a sample of the harvested monovalent virus bulk undergoes sterility testing and may be subject to further PCR testing. The influenza virus is inactivated by incubation with β-propiolactone (BPL) and the residual inactivating agent is then hydrolysed at an elevated temperature. The monovalent bulk harvest is further processed via chromatography for purification and concentration. Subsequent processing steps include detergent treatment to disrupt the viral envelope, followed by separation and extraction of the surface antigen subunits from the viral core. Subsequent purification steps are employed for polishing and concentration of the virus subunits, and the three monovalent vaccines are then blended to produce the final trivalent vaccine formulation.

2.2. Studies supporting risk assessment

2.2.1. Virus growth studies in MDCK 33016 cells

Growth studies using potential adventitious agents were carried out as a prerequisite to the specific risk calculations. Viruses studied included: three human adenovirus (types 1, 5 and 6), herpes simplex virus (HSV), Epstein–Barr virus, cytomegalovirus, parainfluenzavirus 3 and SV-5 parainfluenzavirus, respiratory syncytial virus (RSV) types A and B, human coronavirus E229, human enterovirus species (Coxsackie A16, Coxsackie B30, Echovirus 6, and poliovirus type 1), three different rhinoviruses, mammalian reovirus-3, BK polyomavirus, Simian virus 40 (SV40), budgerigar fledgling disease polyomavirus, avian C-type retrovirus (*Rous sarcoma* virus), avian birnavirus (infectious bursal disease virus [IBDV]), avian reovirus, minute virus of mice (MVM) parvovirus and porcine circovirus. Furthermore, the growth of *Mycoplasma hyorhinis* (a cell culture contaminant strain isolated from a rhinovirus isolate), and *Chlamydia trachomatis* were assessed. The virus strains

used were obtained from reputable sources, including the American Type Culture Collection or specialized academic laboratories, and were genetically or serologically characterized. Where possible, the identity of human adventitious agents was confirmed by independent PCR tests, with the exception of mammalian reovirus. These PCR tests were performed by the Max von Pettenkofer Institute (Munich, Germany).

All growth studies were carried out using a standard experimental scheme: MDCK 33016 suspension cells were grown in the same medium used for vaccine production in 100 ml spinner flasks. Cell suspensions were inoculated with 10^6 tissue culture infectious dose 50% ($TCID_{50}$) of the test virus. Samples of the inoculated suspension cultures (medium plus cells) were taken directly after infection and were frozen and then thawed to measure the inoculum titre by standard virus titrations in specific sensitive cell lines or in primary cells. Further samples were taken in the same way at regular intervals for up to 2–3 weeks after inoculation in order to monitor virus titres. The sample volume was chosen specifically to keep cell densities below a level that would inhibit further cell growth, and the volume removed was replaced with the same volume of fresh medium. All virus titrations were performed using replicates of the titration test culture ($n = 8$) for each $\log_{10}$ dilution step. Virus titres were calculated using the Spearman–Karber formula. Repeatability and intermediate precision of the virus titrations typically varied by a standard deviation of $<0.3\ \log_{10}$.

2.2.2. Virus inactivation and removal studies

Inactivation and removal studies were carried out as a prerequisite to the specific risk calculations. The BPL inactivation process was studied with the same viruses listed above (with the exception of cytomegalovirus), and with various influenza vaccine virus strains, a second parvovirus (canine parvovirus) and also a second herpesvirus (pseudorabies virus). Furthermore, strains of porcine parvovirus, avian retrovirus and avian birnavirus were studied. To generate a worst-case scenario, cell-free but otherwise unpurified cell culture harvests were passed through an identical BPL inactivation process, as used for the influenza vaccine virus during the vaccine manufacturing process. Virus titres were measured before and after inactivation to calculate the virus titre reduction achieved by the inactivation process. The 95% confidence intervals (CI_{95}) of the virus reduction rates ranged between $0.2\ \log_{10}$ and $0.5\ \log_{10}$. For influenza virus, extra studies were carried out to assess separately the inactivation capacity of the BPL hydrolysis step. For this purpose, a 10% volume of infectious virus was spiked into the process material after the initial inactivation step had been performed. Remaining virus titres were measured during and after the hydrolysis step.

Further inactivation and removal studies were carried out for the subunit splitting and purification processes. Virus selection for those studies was governed both by risk of occurrence and the need to provide broad representation of viruses in terms of relevant taxonomic, morphological, and physicochemical characteristics. Reovirus and HSV are relatively stable representatives within the spectrum of non-enveloped and enveloped viruses that are considered in the risk assessment and also represent likely contaminants that may replicate in MDCK 33016 cells as well as in embryonated eggs used for influenza virus isolation. Murine leukaemia virus (MuLV) was also tested, since virus-containing retrovirus models are commonly used and recommended for such studies. Furthermore, simian virus 40 (SV40), a very stable polyomavirus, was also studied. It should be noted, however, that no polyomavirus or polyomavirus sequence, nor retrovirus or reverse transcriptase activity, was detected in MDCK 33016 cells. The viruses were spiked into process material, which was then subjected to the detergent splitting, and subsequent subunit separation and purification steps. Samples were taken to monitor residual virus titres and virus reduction rates were calculated. The amount of virus added to the starting material was carefully chosen to be as high as possible in order to determine the capacity of the process step to inactivate/remove viruses adequately, whilst at the same time not changing the composition of the production material significantly. Reproducible virus reduction was shown by two independent studies, observing published guidance on the viral safety evaluation of other biotechnology products derived from cell lines of human or animal origin [15].

2.3. Risk assessment

The quantitative risk assessment is based on calculating potential adventitious agent exposure amounts that could theoretically be present at any point in the cell culture-derived influenza vaccine manufacturing process, from the initial isolation of the influenza virus strain in eggs through to blending of the final trivalent vaccine. The exposure amounts for potential adventitious agents are estimated in terms of infectious units per ml and are based on published data and on data derived from our own studies (Table 1). Specific virus titre data were used wherever possible (e.g. for growth of adventitious agents in MDCK 33016 cells, limits of detection of PCR-based virus exclusion assays, and for log reduction rates measured during virus inactivation and detergent splitting or separation (Section 2.2)). Worst-case scenarios were assumed where the risk has not previously been documented. The assumptions made for each step of the vaccine production process, and the data on which these assumptions are based, are outlined below.

2.3.1. Throat sample

A maximum titre of $9\ \log_{10}$ infectious units/ml was assumed for any viral agent that might be found in original clinical samples, as from our own experience this is the greatest virus titre that may be harvested from optimal cell culture. During the virus isolation process, the clinical sample is diluted at least 1:10 in a suitable buffer or transport medium of which a 100 μl sample is inoculated into an egg. Taking into account these dilution steps, a maximum of $7\ \log_{10}$ infectious units/ml was assumed as the starting virus titre for any potential contaminant in the original clinical sample.

2.3.2. Virus isolation

Based on the assumptions that a minimum of three egg passages are performed during influenza virus isolation and characterisation, and that each passage uses a dilution factor of 1:100, a titre of $1\ \log_{10}$ infectious units/ml was calculated for any agent that does not grow in embryonated eggs. If growth in eggs cannot be excluded, but is restricted to a well-adapted virus and is expected to be slow and/or ineffective, a titre of $4\ \log_{10}$ infectious units/ml was assumed. This was increased to $6\ \log_{10}$ infectious units/ml if the growth of a particular agent in embryonated eggs cannot be excluded. For all avian viruses and other virus types that are known to grow well in embryonated eggs, a maximum virus titre of $9\ \log_{10}$ infectious units/ml was assumed, where 100 μl volume would be transferred to the next step, resulting in a titre of $8\ \log_{10}$ infectious units/ml for such viruses. It should be noted that much higher dilutions and repeated infection passages are often utilized in practice that would reduce or exclude any contaminant much more effectively than estimated by the assumptions outlined here.

Table 1
Outline of data and assumptions, including worst-case scenarios where necessary, for each step of the manufacturing process

Process step	Data and assumptions used
Throat sample	Worst-case scenario: 9.0 $\log_{10}$ $TCID_{50}$/ml diluted 1:10, 100 μl inoculated, resulting maximum titre of 7 $\log_{10}$ $TCID_{50}$/ml
Virus isolation	≥3 passages using 100 μl inoculum per 10 ml egg allantoic fluid; corresponds to 3× 1:100 dilutions. Consider growth/no growth
MDCK viral-seed passages	≥3 passages at minimum 1:100 inoculum dilution. Consider growth/no growth (from virus growth studies). Intermittent contamination of 4 $\log_{10}$ $TCID_{50}$/ml assumed for all human agents that do not grow in MDCK cells (see Section 2.3.3)
PCR testing of MDCK 33016 viral seeds	Titre reduced to limit of detection. If PCR is not routinely conducted or if calculated titres are already below the limit of detection of the method use residual MDCK virus titre of previous step
Bioreactor inoculum	Worst-case scenario: inoculum dilution 1:1000
Fermentation	Viruses that do grow in MDCK cells: measured maximum titre 3 days after infection Viruses that do not grow in MDCK cells: no further titre reduction
Chromatography steps	Minimum average reduction: 0.5 $\log_{10}$ reduction/ml
Concentration (total process concentration)	Overall process concentration: 1.2 $\log_{10}$ addition/ml
Inactivation	Use data from inactivation studies
Splitting/subunit inactivation	Use data from virus removal studies: reovirus and related viruses: 7.6 $\log_{10}$ reduction Other non-enveloped viruses: 4.8 $\log_{10}$ reduction Herpesviruses: 5.5 $\log_{10}$ reduction Other enveloped viruses: 5.8 $\log_{10}$ reduction
Formulation	Worst-case assumption 0.3 $\log_{10}$ reduction/ml
Human infectious doses per vaccine dose	Assumption: 100 *in vitro* infectious units needed to establish infection in humans

MDCK, Madin–Darby canine kidney; PCR, polymerase chain reaction; $TCID_{50}$, tissue culture infectious dose 50%.

2.3.3. MDCK 33016 cell culture

Assumptions relating to the behaviour of adventitious viruses in MDCK 33016 cell culture were based on data derived from viral growth studies (Section 2.2.1) and supplemented with data extracted from the scientific literature [16–25]. During generation of the MDCK 33016 viral seed, a minimum of three passages was assumed, each one using a minimum virus dilution of 1:100. Again, where MDCK 33016 cells cannot support growth of such viruses, this would result in a virus titre reduction of 6 $\log_{10}$ infectious units/ml of any virus present in the cell culture. However, as stable human agents unable to grow in MDCK 33016 cells may contaminate seed virus passages during open steps carried out by human operators, it was necessary to assume and calculate the possibility of intermittent contamination during these operations. We assumed a contamination of 10^8 infectious units/ml via aerosols or contaminated equipment, of which a total volume of 10 μl infects the smallest culture volume used (100 ml). This results in a final virus content of 4 $\log_{10}$ infectious units/ml culture fluid where, in a worst-case scenario, the virus titre could remain stable without reduction by degradation or by further passage dilutions, particularly if the contamination occurred during the last culture step. This residual virus titre of 4 $\log_{10}$ infectious units/ml was also assumed for all human viruses and for agents for which no specific data were available but where growth in MDCK 33016 cells would be highly unlikely. For viruses that can grow in MDCK 33016 cells, specific titres that were measured during viral growth studies described previously (and carried out under identical conditions as those applied for MDCK 33016 culture passages with influenza virus) were used (Section 2.2.1).

2.3.4. PCR testing of MDCK 33016 viral seeds

The PCR limits of detection, measured in terms of $\log_{10}$ infectious units/ml were applied for all viruses where a specific PCR test was included in the calculations. If no PCR test was included in the calculation, or if the calculated titre of the viral seed was already reduced to below the detection limit of the PCR test, the residual virus titres calculated before this step were maintained.

2.3.5. Bioreactor inoculum

During the bioreactor inoculation step, at least a 1:1000 dilution of the seed virus was assumed to occur when used to inoculate the large-scale bioreactor cell culture. This would result in a reduction of any adventitious virus of at least 3 $\log_{10}$ infectious units/ml. In practice, higher dilutions are normally used.

2.3.6. Fermentation

Maximum residual titres, measured after 3 days' fermentation, were applied for all viruses that do grow in MDCK 33016 cells (Section 2.2.1). For all agents that do not grow in MDCK 33016 cells, or are unlikely to grow in MDCK 33016 cells, a worst-case scenario of no titre loss was assumed, and residual titres from the previous step were carried forward. Where PCR testing of bioreactor harvests was included in the calculations, the relevant detection limit of the PCR test was taken as the post-fermentation titre.

2.3.7. Chromatography steps

Chromatography steps that are carried out to purify and concentrate influenza viruses may also concentrate other viruses, resulting in higher virus titres in the concentrated intermediate product. To generate specific data for these process steps, virus-spiked raw products were processed through the same chromatography steps, and virus titres remaining in the intermediate product were measured. The chromatography steps did not lead to any increase in virus concentration for any of the three viruses tested (HSV, MuLV, reovirus 3); in fact virus titre reduction was observed. In the risk assessment, the lowest average sum of reductions was applied to all viruses. This was a 0.5 $\log_{10}$ infectious units/ml reduction, as observed for reovirus.

2.3.8. Concentration (total process concentration)

To calculate the maximum degree of virus concentration that could occur during the purification process, the lowest yields of various influenza virus strains observed during development of the vaccine manufacturing process were used to calculate the amount of virus harvest needed to produce 1 ml of a monovalent bulk concentrate. For the lowest yields observed, a 17-fold (1.2 $\log_{10}$)

concentration would be required to arrive at a potent monovalent concentrate. Therefore, for all viruses assessed, a concentration factor of 1.2 $\log_{10}$ was included in the calculation to account for the maximum possible concentration of virus that could occur. This factor represents the total effect of several downstream dilution and concentration steps.

2.3.9. Inactivation

The calculations of maximum residual adventitious virus titres, after the BPL inactivation step, were based on data derived from inactivation studies with relevant viruses (Section 2.2.2) and, where necessary, on a representative selection of virus strains and subtypes. If more than one definite value was available for virus types of the same taxonomic group, the lowest virus titre reduction was used (defined values are measured as the result of an incomplete inactivation). If several virus types from the same group showed complete inactivation (i.e. reduction rates were greater than or equal to the measured values), the highest virus titre reduction measured was employed. In some cases, inactivation rates were taken from related viruses; for example, calculations for mumps virus and measles virus were deduced from (lowest) values measured for other members of the paramyxoviridae; human retroviruses were calculated with data obtained for an avian retrovirus; human hepatitis viruses B, C and G inactivation rates were assumed to be similar to the relatively resistant HSV; and avian circovirus was assumed to be equally well inactivated as porcine circovirus.

2.3.10. Splitting and subunit purification

Residual virus titres following the detergent splitting and subunit purification stage were based on data from studies on a representative selection of viruses (Section 2.2.2). The two enveloped viruses, HSV and MuLV, were completely inactivated during the detergent treatment step, with reduction rates of $\geq$5.5 to $\geq$5.8 $\log_{10}$ infectious units/ml reported after the detergent treatment step. Non-enveloped viruses were less affected by the detergent treatment, but more affected by the subsequent separation process, with a reduction of $\geq$7.6 $\log_{10}$ steps reported for reovirus and 4.8 $\log_{10}$ steps for SV40. The CI_{95} of the virus reduction rates ranged between 0.2 $\log_{10}$ and 0.3 $\log_{10}$. For the purpose of the risk assessment, a reduction of 4.8 $\log_{10}$ infectious units/ml was assumed for all non-enveloped viruses, except reovirus and closely related birnavirus, where a reduction of 7.6 $\log_{10}$ was applied. A reduction of 5.5 $\log_{10}$ infectious units/ml was assumed for herpesviruses, and a 5.8 $\log_{10}$ reduction for all other enveloped viruses, knowing that both values would grossly underestimate the true reduction should contamination by an enveloped virus occur.

2.3.11. Formulation

As the trivalent subunit influenza vaccine is supplied in a 0.5 ml dose, a maximum of 0.167 ml of each monovalent bulk is used to formulate the final trivalent subunit vaccine. This would correspond to a reduction of any viral contaminant by 0.778 $\log_{10}$. If, however, each of the three combined monovalent bulks contained the same amount of adventitious virus, then the corresponding volume reduction would be 1/2 or 0.33 $\log_{10}$. Although formulation of the final product is normally associated with dilution to adjust the vaccine to the correct antigen concentration/potency, no such dilution was taken into consideration, and for the purpose of the risk assessment, a worst-case assumption of 0.3 $\log_{10}$ reduction/ml was made for all viruses tested.

2.3.12. Human infectious doses per vaccine

Infectious doses as measured in sensitive *in vitro* systems normally surpass animal (and human) infectious doses by orders of magnitude. Correlations between *in vitro* titres and human infectious doses can best be deduced from live vaccines (mumps, measles, rubella, varicella zoster or polio vaccines), which must contain a minimum *in vitro* titre of between 1000 and 20,000 or >300,000 infectious units to reliably establish infection in the vaccinated individual. For the purposes of this risk assessment, a minimum of 100 *in vitro* infectious units were assumed to be sufficient to establish infection in humans, as specific data for each of the agents tested in this study do not exist. For any virus that is not known to infect humans, for example, avian reovirus, this calculation may be inadequate, but as there is sufficient evidence in only a very few cases to absolutely exclude human infections, it was decided to calculate all final virus titres by the same method.

3. Results

3.1. Virus growth studies in MDCK 33016 cells

Risk assessments and appropriate countermeasures should concentrate on those agents that are able to replicate in MDCK cells. Extensive testing of a wide range of viruses has been performed to determine their ability to grow in MDCK cells, and to ascertain their relevance as a potential risk from the perspective of an inactivated MDCK cell culture-derived influenza vaccine (Table 2). These studies have demonstrated the ability of MDCK 33016 cells to support the growth of a limited number of human viruses; HSV, parainfluenza 3 and mammalian reovirus 3 can grow efficiently in MDCK 33016 cells, in agreement with published data on MDCK cells [16–18]. HSV and parainfluenza 3 grew to high titres of up to 8 $\log_{10}$ infectious units/ml, whereas reovirus 3 grew slowly and

Table 2
Summary of virus growth in MDCK 33016 cells grown in serum-free medium

Virus family	Virus type (strain)	Growth
Paramyxoviridae	Parainfluenzavirus 3 (C 243)	High growth
	Simian virus 5	High growth
	Respiratory syncytial virus A (A2)	None
	Respiratory syncytial virus B (B)	None
Picornaviridae	Echovirus type 6 (D'Amori)	None
	Coxsackie virus A16 (G10)	None
	Coxsackie virus B30 (Nancy)	None
	Poliovirus type 1 (Sabin)	None
	Rhinovirus type 1B	None
	Rhinovirus type 37	None
	Rhinovirus Neth. 9501841	None
Coronaviridae	Human coronavirus (E229)	None
Retroviridae	Avian retrovirus (*Rous sarcoma* virus, RAV-1/BH)	None
Reoviridae	Mammalian reovirus type 3 (Abney)	Slow growth
	Avian reovirus (U Conn 1133)	Low growth
	Avian reovirus (GB 120)	Low growth
Birnaviridae	Avian birnavirus (IBDV)	None
Parvoviridae	Minute virus of mice (Crawford)	None
Circoviridae	Porcine circovirus (PCV-2 1010)	None
Herpesviridae	Herpes simplex virus 1 (ET)	High growth
	Human cytomegalovirus (AD 169)	None
	Epstein–Barr virus	None
Adenoviridae	Human adenovirus type 1 (IS 305-90)	None
	Human adenovirus type 5 (IS 154-89)	None
	Human adenovirus type 6 (IS 524-90)	None
Polyomaviridae	Simian virus 40 (EK)	None
	BK polyomavirus (18034)	None
	BK polyomavirus (VR-837)	None
	Avian polyomavirus (BFDV-5)	None
Other agents	*Mycoplasma* spp.	None
	Chlamydia spp.	None

Table 3
Estimations of viral $\log_{10}$ infectious units/ml at each stage of the manufacturing process from initial isolate to a final trivalent influenza vaccine (assuming worst-case conditions)

Process step	Influenza	Adenovirus	Parainfluenza	BK polyomavirus	Avian reovirus
Throat sample	7.0	7.0	7.0	7.0	n/a
Egg isolate and passages	8.0	8.0	8.0	6.0	8.0
MDCK viral seed passages	9.0	4.0[a]	7.9	4.0[a]	6.8
PCR-tested seed	9.0[b]	2.0[c]	3.0[c]	2.0[c]	6.8[b]
Bioreactor inoculum	6.0	−1.0	0.0	−1.0	3.8
Bioreactor harvest	9.0	−1.0	3.0[a]	−1.0	6.0
Chromatographies I and II	8.5	−1.5	2.5	−1.5	5.5
Concentration	9.7	−0.3	3.7	−0.3	6.7
BPL inactivation	−7.8	−2.8	−5.8	−2.4	0.3
Splitting/subunit purification	−13.6	−7.6	−11.6	−7.2	−7.4
Formulation/final product	−13.9	−7.9	−11.9	−7.6	−7.7
Human infectious dose per vaccine dose	−15.9	−9.9	−13.9	−9.5	−9.7

BPL, β-propiolactone; MDCK, Madin–Darby canine kidney; PCR, polymerase chain reaction.
[a] No viral growth, but assume contamination by human operators during the final-stage passage in MDCK 33016 cell culture.
[b] PCR test not included in calculation, therefore the residual titre calculated in the previous step is maintained.
[c] PCR detection limit applied at this step.

to moderate titres. Efforts to grow parainfluenza 1, 2, 4A and 4B are still underway and have not yet shown conclusive results, but data collected to date indicate low or no growth. Published data indicate that mumps virus may grow inefficiently in MDCK cells [19] and the proliferation of well-adapted measles virus strains, albeit with slow growth, has also been reported [20,21]. There are conflicting reports in the published literature concerning the ability of RSV to grow in MDCK cells. Whereas earlier studies have reported increased RSV titres after inoculation in MDCK cells [22,23], others have reported that MDCK cells showed a high degree of resistance to RSV [24]. When evaluating different cell substrates for the isolation of RSV from clinical specimens, only two RSV positive isolates were found in MDCK cells, compared with at least 12, or up to 30, positive isolates in other cell substrates [24]. Our own virus growth studies, monitoring growth of cell culture-adapted RSV-A and RSV-B strains under serum-free conditions in MDCK 33016 suspension cells, did not reveal any evidence of virus growth, and virus titres persistently dropped to below detection limits within 3–5 days. Thus, if possible at all, growth of RSV in MDCK cells seems to be restricted to certain strains and yields only moderate virus titres, especially if MDCK cell cultures are co-infected with influenza virus [23]. MDCK 33016 cells do not support the growth of most avian viruses but may allow replication of avian reovirus to a limited degree. Published studies report an inability of MDCK cells to support avian reovirus propagation [25,26]; however our own replication studies demonstrated minor avian reovirus titre increases after inoculation of MDCK 33016 cells with high titres of avian reovirus. Thus, minor and restricted growth was assumed, although the observed titre increase after inoculation of the test cultures could also be due to the dissociation of viral aggregates.

3.2. Risk assessments

The risk assessment covers a spectrum of adventitious viruses that might be encountered during the cell culture-derived influenza vaccine manufacturing process, but also includes a broad selection of other viral agents representing major viral morphological, taxonomic and physical properties. It includes DNA and RNA viruses, enveloped and non-enveloped viruses, double-stranded, single-stranded or circular DNA/RNA genomes, small or large and complex viral genomes, and viruses with low, intermediate or extremely high stability. The application of the risk-assessment model to influenza virus, and to potential contaminants parainfluenza virus, adenovirus, reovirus and BK polyomavirus, is described in detail below and in Table 3. The potential contaminants represent different scenarios, such as viruses capable of growing in MDCK cells or not, enveloped or non-enveloped viruses and stable or unstable viruses. General process effects, for example, process dilution and concentration steps, apply to all viruses in the same way and so are described below within the context of residual influenza virus titres. Only virus-specific effects of the risk-model will be discussed for the other viruses. Overall estimations of maximum titres per vaccine dose for all the viruses/virus families tested are presented in Table 4.

3.2.1. Influenza virus

The influenza virus is an enveloped virus containing a negative strand RNA genome. In line with the risk-assessment model, a maximum of 9 $\log_{10}$ infectious units/ml was assumed to be present in the original clinical sample. Taking into account inoculum volumes, this titre may be reduced during passaging steps; however, MDCK 33016 cells are highly permissive for influenza virus growth

Table 4
Estimations of maximum human infectious units per vaccine dose for a range of potential adventitious agents

Virus/agent	Human infectious dose per vaccine dose ($\log_{10}$)
Influenza virus	−15.9
Adenovirus	−9.9
Herpes simplex virus	−9.9
Other herpes virus (human herpesviruses 2–8)	−10.0
Parainfluenza	−13.9
Respiratory syncytial virus	−14.8
Mumps/measles virus	−9.8
Coronavirus	−14.0
Rhinovirus	−12.4
Enterovirus	−11.7
SV40, JC/BK polyomavirus	−9.5
Hepatitis B, C, G	−10.9
Human retrovirus	−13.4
Mammalian reovirus	−6.1
Avian retrovirus	−13.6
Avian polyomavirus	−8.9
Avian reovirus	−9.7
Avian birnavirus	−12.1
Avian circovirus	−9.7
Porcine circovirus	−11.7
Minute virus of mice parvovirus	−13.9
Mycoplasma	−13.0
Chlamydia	−13.3

and so a maximum residual titre of 9 $\log_{10}$ infectious units/ml was assumed to be present in the bioreactor harvest. Applying the general risk-assessment assumptions to the chromatography processes and concentration steps, a maximum residual titre of 9.7 $\log_{10}$ infectious units/ml is expected at this point in the manufacturing process. Both the BPL incubation and BPL hydrolysis steps have been assessed for their inactivation potential. The combined results from both steps demonstrated the capacity of the processes to inactivate influenza virus by $\geq$17.5 $\log_{10}$ infectious units/ml, resulting in a maximum residual titre of $-$7.8 $\log_{10}$ infectious units/ml after the inactivation process. A further reduction of $\geq$5.8 $\log_{10}$ infectious units/ml was assumed to occur during the splitting of the viral envelope, based on data derived from studies on the enveloped viruses HSV and MuLV. During the final blending of the trivalent vaccine, this was assumed to be further reduced to a maximum residual titre of $-$13.9 $\log_{10}$ infectious units/ml. As described in the risk-assessment model, this may then be translated into $-$15.9 $\log_{10}$ human infectious doses of influenza per vaccine dose.

3.2.2. Adenovirus

Human adenovirus is a non-enveloped virus with a double-stranded DNA genome. Again, a maximum 9 $\log_{10}$ infectious units/ml was assumed to be present in the original clinical sample and, as adenovirus may grow in eggs, a maximum residual titre of 8 $\log_{10}$ infectious units/ml was assumed to be present in the egg-derived inoculum that is used for subsequent MDCK cultures. As described in Section 2.3.3, contamination by human operators was assumed to occur during the final-stage passage in MDCK 33016 cell culture, and a titre of 4 $\log_{10}$ infectious units of stable virus/ml was assumed to be present in the MDCK-derived viral seed. PCR testing of the viral seed was incorporated into the adenovirus calculation, with a known detection limit of 2 $\log_{10}$ infectious units/ml, indicating the maximum residual titre that could be present at this stage of the manufacturing process. The adenovirus double-stranded genome is relatively resistant to nucleic acid destruction by BPL, as demonstrated by our own inactivation studies on three human adenoviruses and one canine adenovirus where a minimum reduction of 2.45 $\log_{10}$ steps was observed. Again, in our own studies, the splitting and subunit purification steps reduced SV40, a very stable, non-enveloped, double-stranded DNA virus, by 4.8 $\log_{10}$ steps. Applying this to adenovirus, a theoretical maximum residual titre of $-$7.6 $\log_{10}$ infectious units/ml might remain in the purified monovalent bulk at this stage of the manufacturing process. This results in a final maximum residual titre of $-$7.9 $\log_{10}$ infectious units/ml in the final trivalent vaccine, which further translates into a potential $-$9.9 $\log_{10}$ human infectious doses per vaccine dose.

3.2.3. Parainfluenza virus

Human parainfluenza virus is an enveloped virus with a negative strand RNA genome. As parainfluenza virus can grow in eggs, a maximum residual titre of 8.0 $\log_{10}$ infectious units/ml was assumed to be present in the egg-derived inoculum used to inoculate MDCK cells. In our own virus growth studies, parainfluenza 3 has been shown to replicate quickly and to high titres in MDCK cells, with maximum residual titre of 7.9 $\log_{10}$ infectious units/ml observed after 3 days in culture, using cell culture-adapted parainfluenza 3 strains. This value was taken as the maximum residual titre that could theoretically be present in the MDCK seed virus. PCR testing of the working viral seed is routinely carried out and so the theoretical maximum parainfluenza titre that could remain would correspond to the PCR detection limit of the parainfluenza PCR assay, which is 3 $\log_{10}$ infectious units/ml. This value may be increased by process concentration to a maximum of 3.7 $\log_{10}$, but is significantly reduced by BPL inactivation, detergent treatment, and subunit purification. Complete inactivation of two parainfluenza strains by BPL has been demonstrated during our own inactivation studies, where a reduction $\geq$9.5 $\log_{10}$ infectious units/ml was measured. Based on data derived from studies using the enveloped viruses HSV and MuLV, a $\geq$5.8 $\log_{10}$ reduction in parainfluenza titres was assumed to occur during the splitting and subunit purification stages of the manufacturing process. It is highly likely that this value underestimates the real capacity of the purification stages to remove parainfluenza virus. In our own inactivation studies on HSV and MuLV, the first detergent step successfully removed all contaminating virus and so subsequent separation steps could not be assessed in terms of their inactivation potential. Including subsequent manufacturing steps, a theoretical maximum titre of $-$11.9 $\log_{10}$ infectious units/ml could remain in the cell culture-derived vaccine, should a parainfluenza contamination event occur. According to the assumptions defined above, this further translates into a potential $-$13.9 $\log_{10}$ human infectious doses per vaccine dose.

3.2.4. BK polyomavirus

BK polyomavirus is an extremely stable, non-enveloped virus with a double-stranded DNA genome. The virus is widespread in nature and after primary infection is likely to remain latent in immunocompetent subjects, but is potentially pathogenic in immunocompromised individuals. BK polyomavirus serves as a good model for assessing viral safety, as it is a particularly stable virus that is extremely difficult to inactivate or remove from contaminated material. No data are available on the permissiveness of embryonated eggs for BK polyomavirus and so a maximum of 6 $\log_{10}$ infectious units/ml was assumed. BK polyomavirus did not grow in MDCK 33016 cells in our experiments, but intermittent contamination was assumed to occur during the final-stage passage in MDCK 33016 cell culture, resulting in a titre of 4 $\log_{10}$ infectious units/ml (see Section 2.3.3). The PCR detection limit for BK polyomavirus is $\leq$2 $\log_{10}$ infectious units/ml; therefore, the maximum amount of BK polyomavirus that could theoretically remain in the MDCK influenza seed virus is 2 $\log_{10}$ infectious units/ml. In line with other double-stranded genome viruses, for example herpes, reovirus or birnavirus, BK polyomavirus is very resistant to chemical inactivation. Accordingly, BPL caused a relatively small reduction of 2.05 $\log_{10}$ steps. Assumptions regarding the removal of BK polyomavirus during the subunit purification stages of the manufacturing process were based on data derived from our own virus-spiking studies carried out on SV40 where a virus titre reduction by 4.8 $\log_{10}$ was observed. Applying this to BK polyomavirus, and taking into account subsequent manufacturing steps, a maximum residual titre of $-$9.5 $\log_{10}$ human infectious doses per vaccine dose could theoretically remain in the vaccine should a contamination event occur.

3.2.5. Avian reovirus

Avian reovirus is a non-enveloped double-stranded RNA virus, the growth of which is supported by embryonated eggs. Although avian reovirus would not be present in the original clinical isolate, a maximum titre of 8 $\log_{10}$ infectious units/ml was assumed in the egg isolate, as this virus could be present in the embryonated eggs used for influenza viral strain isolation. Previous studies have not demonstrated growth of avian reovirus in MDCK cells [27], but our own studies have shown titre increases in avian reovirus when inoculated at high titres into trypsin-containing MDCK 33016 cell cultures (5.2 $\log_{10}$ infectious units/ml rising to 6.75 $\log_{10}$ infectious units/ml). Although it is unclear whether the

titre increases are due to virus growth or are a result of dissociated virus aggregates, a viral titre of 6.8 $\log_{10}$ infectious units was assumed for repeated seed virus passages in MDCK cells. For the bioreactor harvest, the maximum titre increase as observed for a high virus titre inoculum after 3 days in culture was taken as the resulting end value. No titre reduction due to PCR testing for avian reovirus was calculated. Our own inactivation studies demonstrated a 6.4 $\log_{10}$ infectious units/ml reduction in avian reovirus (U/Conn 1133 prototype strain) titres following treatment with BPL. Complete inactivation was also achieved with another field isolate of an avian reovirus. This was a much greater inactivation than that seen with BPL treatment of mammalian reovirus 3. Virus reduction by the subunit purification process of $\geq$7.6 $\log_{10}$ infectious units/ml as measured for reovirus 3 was also applied to avian reovirus and so a maximum of −7.4 $\log_{10}$ infectious units/ml could theoretically be present at the end of the purification processes. This translates into a worst-case scenario of −7.7 $\log_{10}$ infectious units/ml being present in the final trivalent vaccine or −9.7 $\log_{10}$ human infectious doses per final trivalent vaccine dose, although avian reovirus is not known to infect humans.

3.2.6. Other viruses and agents

When applying the risk assessment to various other viruses/virus families, maximum infectivity titres of between −6 $\log_{10}$ and −16 $\log_{10}$ infectious units per vaccine dose were calculated (Table 4). Lack of growth in both embryonated eggs and MDCK 33016 cells was assumed for human herpes viruses 6, 7 and 8; human hepatitis viruses B, C and G; and human retroviruses, based upon current knowledge about the restricted host spectrum of these viruses and confirmed by negative results from a systematic literature search. PCR testing was not included in the calculations for these viruses and inactivation rates were based on data obtained for the closest model virus; lowest inactivation rates obtained for different herpesviruses were also applied to other herpesviruses and to hepatitis B, C and G viruses, and inactivation rates measured for an avian retrovirus were applied to human retroviruses.

Porcine circovirus could potentially contaminate the vaccine process stream if trypsin of porcine origin were to be used in the manufacturing process. These small and stable viruses may be viewed as 'worst-case' contaminants in a vaccine manufacturing process and so were included in the risk assessment. Porcine circovirus was also used as a model for the avian circoviruses. Avian circovirus demonstrates a strict host-cell tropism, and based on the information available, limited growth in embryonated eggs and no growth in MDCK cells was assumed [27,28]. As a result of previous reports of contamination from an unknown source during large-scale fermentation of rodent cells [29], MVM parvovirus was also included in the studies and in the model calculations.

The risk assessment has also been applied to two bacterial pathogens, *Mycoplasma* and *Chlamydia*. *Mycoplasma* is a common cell culture contaminant and it is important to assess what effect the manufacturing process would have in the event of a contamination. It should be noted that growth studies revealed that *Mycoplasma* (an *M. hyorhinis* strain isolated as a cell culture contaminant from a rhinovirus isolate) was unable to grow in the chemically defined, serum-free MDCK 33016 culture used for seed-lot passages and fermentation, but grew rapidly and to high titres when low amounts of serum were added to the cultures. As *Chlamydia* contamination cannot be detected by common bacterial sterility tests, it was deemed necessary to also assess and calculate the effects of such a contamination. Based on data derived from growth studies and inactivation studies, the specific manufacturing process is capable of reducing both agents to $\leq$−13 $\log_{10}$ human infectious doses per vaccine dose.

4. Discussion

Influenza viral seeds are produced by the WHO on an annual basis to match drifting influenza strains [1]. Reference viruses are released to vaccine manufacturers after the WHO recommendations have been published and the vaccine must be manufactured, tested and distributed within only a few months in order to meet vaccination schedules [2]. Because of this short timeline, conventional broad-spectrum testing of the viral seeds for adventitious agents cannot be performed before manufacturing commences, and may not even be completed before the distribution of the final trivalent vaccine. Thus, any new inactivated influenza vaccine manufacturing process should be designed to remove any potential contaminants introduced via the viral seed. Extensive viral testing of the MDCK cells themselves is part of the standard process of cell substrate evaluation and did not reveal any contaminant in the cell line. Furthermore, measures were developed to ensure that safety margins exceeded any potential level of contamination. Such measures include the virus inactivation process, rapid testing for specific virus detection (e.g. PCR testing) and proof of effective virus removal by the detergent splitting and subunit purification process.

Before the contamination risk of a cell culture-derived vaccine manufacturing process can be assessed, it is necessary to determine which viruses can actually grow in the cell substrate in order to determine the relevance of such agents in the context of contamination risk. Some data have been derived from the published literature, but this is not a comprehensive resource and so replication studies have been carried out to assess the growth characteristics of >20 viruses/virus families in MDCK cells. Data derived from replication studies confirm that MDCK 33016 cells support the growth of only a limited range of viruses (in this context the relevant viruses are influenza virus, parainfluenza virus, reovirus, and herpes simplex virus), similar to those viruses that can grow in eggs. Therefore, like embryonated eggs used in current influenza vaccine manufacture, MDCK 33016 cells can act as an effective viral filter for a wide range of adventitious agents. Moreover, MDCK 33016 cells do not support the replication of many avian viruses. This is of particular relevance if an avian virus contaminant is introduced into the process by passaging the vaccine virus strain in eggs. Therefore, using MDCK cells for virus propagation can lower the product- and process-failure risk. Conversely, for those viral agents that have the ability to grow well in MDCK 33016 cells, namely parainfluenza virus, HSV and reovirus, further consideration is needed.

Using the model described here, we have assessed the ability of the cell culture-derived influenza vaccine manufacturing process to reduce a range of potential contaminants to a level far below an infectious dose. A maximum titre of between 10^{-6} and 10^{-16} $\log_{10}$ residual infectious units per dose of MDCK-based vaccine has been calculated for all relevant potential adventitious agents. These figures do not indicate a risk factor; for example, an end result of 10^{-6} $\log_{10}$ does not mean that one in one million vaccine doses will contain enough virus to infect a human being. Instead, the data refers to infectious units, and so indicates the level of virus to be anticipated in the manufactured vaccine. In other words, if a contamination event were to occur in the vaccine manufacturing process, theoretically, >1 million doses of that batch of vaccine would have to be administered to one individual to deliver an infectious dose.

Any variability in the data used in the calculations described here will have an impact on the outcome of the assessment. Our

studies have demonstrated an average CI_{95} of 0.3 $\log_{10}$ infectious units/ml for independent virus inactivation and virus removal steps, and a similar CI_{95} for virus growth titres, suggesting that the combined variability is unlikely to exceed 0.99 $\log_{10}$ infectious units/ml for all 11 manufacturing steps considered in this assessment (assuming similar variability at each stage of manufacture). Virological differences will also impact on the assessment outcome; SV40 is commonly used as a model virus for other polyomaviruses but in our studies the BPL inactivation rate of SV40 exceeded that measured for both BK polyomavirus and avian polyomavirus by 1.0–1.4 $\log_{10}$ steps. Similarly, avian reoviruses were highly sensitive to BPL inactivation, whereas mammalian reovirus, and the related birnavirus, were much more resistant (the difference in inactivation rates ranged from $\geq$2.8 to $\geq$4.5 $\log_{10}$ steps). Inactivation rates for the enteroviridae also varied, with differences of 2–3 $\log_{10}$ steps observed. Consistent inactivation rates were observed for the adenovirus, rhinovirus and herpesvirus families and some of the paramyxoviridae family.

Should a contamination ever be detected, the risk calculations presented here could also be used to predict the consequences and actions required to address such an event. In the best case, these calculations and the underlying studies may be used as convincing evidence that no action is needed because the process is able to remove and inactivate the contaminant. This can be of critical relevance for influenza vaccines where a seed virus strain cannot be readily exchanged by another strain, and when time-consuming measures to remove adventitious agents may no longer be possible if a contamination is detected late during the annual vaccine-manufacturing period. Modelling the whole process may also help to identify where virus exclusion tests are needed, for example, for those viruses that grow to high titres and cannot be adequately removed or inactivated. Moreover, virus titres calculated for each relevant step of the entire process can also indicate where such exclusion tests are useful and where they are not. For example, from Table 3 it can be seen that an exclusion test for adenovirus would not be appropriate for the modelled process if it were to be conducted at the bioreactor harvest stage, or further downstream, because the expected worst-case viral titre would be below the method's detection limit. Conversely, the calculations show that testing for reoviruses may be useful to identify any contaminating reovirus, although the comfortably high safety margins of the risk assessment indicate that such measures are not absolutely necessary. The calculations presented here validate the capacity of the cell culture-derived influenza vaccine manufacturing process to remove and/or inactivate any type of potential contaminant, and it can be concluded that the risk of exposure to adventitious agents in vaccines produced in MDCK 33016 cells, using the specific manufacturing processes, is negligible.

As current influenza vaccine production incorporates exclusion tests for avian retrovirus only, relying on the filter effect of embryonated eggs, the risk-assessment model described here provides a more controlled and safeguarded method for assessing the potential impact of adventitious agents in the manufacture of trivalent subunit influenza vaccines.

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