EVALUATION OF AVIAN ADENOVIRUS INACTIVATION METHODS USED IN THE PRODUCTION OF INFLUENZA VACCINES

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Inactivation of influenza virus and other potential contaminants like avian adenoviruses coming from embryonated chicken eggs is a critical step in the production of inactivated influenza vaccines. Inactivation must lead to a guaranteed reduction in contaminant titers by at least 4 lg (PFU)/ml. The aim of this study was to identify an optimum cell line for adenovirus propagation and to estimate a reduction in adenovirus titers in vaccine intermediates after inactivation. In a series of experiments, we identified the optimum conditions and the optimum cell line for the propagation of avian adenovirus (strains CELO and Fontes). The most commonly used inactivation methods were analyzed, including inactivation by β-propiolactone and UV light. Viral titers were measured by plaque assays. After 10 h of inactivation with β-propiolactone, CELO titers fell by 4.12 ± 0.06 lg, whereas Fontes titers, by 4.20 ± 0.19 lg, suggesting that β-propiolactone is an effective inactivating agent. Exposure to UV light led to a reduction in CELO titers by 4.69 ± 0.89 lg and a reduction in Fontes titers by 4.44 ± 1.06 lg after 5 min. N-octyl-β-D-glucopyranoside added at the splitting step reduced CELO titers by 0.93 ± 0.15 lg and Fontes titers by 1.04 ± 0.12 lg, whereas tetradecyltrimethylammonium bromide led to a reduction in CELO and Fontes titers by 1.18 ± 0.17 lg and 1.12 ± 0.38 lg, respectively.

Keywords: influenza vaccine, inactivation, avian adenovirus, propiolactone, UV radiation

Author contribution: all authors have equally contributed to the methodology of the study, analysis and interpretation of the results and manuscript preparation.

Compliance with ethical standards: the study complied with the principles of the Declaration of Helsinki (1964) and its revisions.

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ОЦЕНКА МЕТОДОВ ИНАКТИВИРОВАНИЯ АДЕНОВИРУСА ПТИЦ ПРИ ПРОИЗВОДСТВЕ ГРИППОЗНЫХ ВАКЦИН

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При производстве инактивированных гриппозных вакцин на стадии инактивации должен быть инактивирован как вирус гриппа, так и возможные вирусные контаминанты (например, аденовирус птиц), которые могут попасть в вакцину из сырья (куриных эмбрионов). Инактиваторы должны обеспечивать гарантированное снижение вирусной нагрузки контаминанта не менее чем на 4 lg (БОЕ)/мл, что обеспечит его отсутствие в готовой вакцине. Целью работы было выбрать клеточную линию для наработки аденовируса и оценить снижение титра аденовируса в полупродуктах гриппозных вакцин при воздействии инактиваторов. Были подобраны оптимальные условия наработки аденовируса птиц штаммов CELO и Fontes в культуре клеток, в качестве оптимальной выбрана культура клеток Vero; рассмотрены основные используемые инактиваторы: β-пропиолактон и УФ-излучение. Титры аденовируса определяли методом бляшкообразования. Спустя 10 ч инактивации β-пропиолактоном аденовирус штамма CELO показал снижение вирусной нагрузки на 4,12 ± 0,06 lg, а аденовирус штамма Fontes — на 4,20 ± 0,19 lg, что указывает на эффективное действие β-пропиолактона при инактивации. Проведение инактивации УФ-излучением позволяет снизить вирусную нагрузку штамма CELO на 4,69 ± 0,89 lg, а штамма Fontes — на 4,44 ± 1,06 lg за 5 мин. Отмечено, что добавление детергента на стадии расщепления также снижает вирусную нагрузку на 0,93 ± 0,15 lg и 1,04 ± 0,12 lg для штаммов CELO и Fontes соответственно при использовании н-октил-β-D-глюкопиранозида и на 1,18 ± 0,17 lg и 1,12 ± 0,38 lg при использовании тетрадецилтриметиламмоний бромида.

Ключевые слова: гриппозные вакцины, инактивация, аденовирус птиц, пропиолактон, УФ-излучение

Вклад авторов: все авторы внесли равнозначный вклад в разработку методики исследования, получение, анализ и интерпретацию данных, в написание и редактирование статьи.

Соблюдение этических стандартов: исследование проведено с соблюдением этических принципов Хельсинкской декларации Всемирной медицинской ассоциации 1964 г. и последующих ее пересмотров.

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One of the key steps in the production of inactivated influenza vaccines is influenza virus inactivation for the safety of the final product. The World Health Organization and the European Medicines Agency [1, 2] require that influenza virus should be completely inactivated during this step. It is known that vaccine intermediates can potentially contain other contaminants like avian leukosis virus, avian adenoviruses and mycoplasmas. So, the guidelines prescribe that the inactivation step should be effective against these pathogens, too.

Technologically, inactivation can be achieved by physical or chemical methods. The most widespread physical method is irradiation with ultraviolet light; one of the commonly used chemical methods is exposure to alkylating agents, such as β-propiolactone [3].

Avian adenoviruses cause a chronic infection in birds and are lethal for chicken embryos (CE). Avian adenoviruses are members of the Aviadenovirus genus. So far, 12 serologically distinct types of avian adenoviruses from CELO (Chicken

Embryo Lethal Orphan) and GAL (Gallus Adeno-Like) virus groups are recognized; one more serotype is represented by the causative agent of the egg drop syndrome (EDS-76) [4].

In chickens, adenovirus infection manifests as inclusion body hepatitis, hepatitis-hydropericardium syndrome, gizzard erosion, respiratory conditions, growth retardation, and joint inflammation [5].

Avian adenovirus infection often develops as a secondary infection in poultry with infectious bronchitis, mycoplasma infection and other respiratory diseases.

Now and then, outbreaks of avian adenovirus infection occur on poultry farms across Russia [6, 7].

According to the literature, adenoviruses can be inactivated with formaldehyde [8]; however, the efficacy of this method has been tested on influenza virus and adenoviruses propagated in MDCK cells; therefore, the results cannot be extrapolated to the egg-based technology of influenza vaccine production. It is known that formaldehyde reduces the immunogenicity of the final vaccine to a much greater extent than β-propiolactone; besides, β-propiolactone inactivates influenza more effectively [9].

As an inactivating agent, β-propiolactone is preferred over formaldehyde because β-propiolactone hydrolyzes to 3-hydroxypropionic acid, an intermediate product of lipid metabolism in humans [10]; this has a beneficial effect on vaccine safety.

The aim of this study was to find an optimum virucidal agent for the inactivation of influenza vaccine contaminants (CELO and GAL viruses) and to determine the minimum inactivation time needed for a guaranteed reduction in viral titers by at least 4 lg (PFU)/ml [11].

METHODS

Material

The avian adenovirus from the Adenoviridae family, Aviadenovirus, Fowl adenovirus A, Fowl adenovirus 1, strain: Phelps (CELO), ATCC VR-432 (ATCC collection; USA).

The avian adenovirus from the Adenoviridae family, Aviadenovirus, Fowl adenovirus D, Fowl adenovirus 2, strain: Fontes, ATCC VR-280 (ATCC collection; USA).

HEp-2 cells (collection of cell cultures of Saint Petersburg Pasteur Research Institute of Epidemiology and Microbiology; Russia).

MA-104 cells (collection of cell cultures of Saint Petersburg Pasteur Research Institute of Epidemiology and Microbiology; Russia).

Vero cells (collection of cell cultures of Saint Petersburg Pasteur Research Institute of Epidemiology and Microbiology; Russia).

Cultivation of CELO and Fontes viruses and measurement of infectious titers

The optimum cell culture for the propagation of adenoviruses was selected from 3 candidate cell lines: Vero B, MA-104 and HEp-2. The cells were cultured in the alpha-MEM growth medium supplemented with Gibco's heat-inactivated fetal bovine serum (10%), 2 mM L-glutamine and 100 μg/ml gentamicin. The maintenance medium used in the experiment contained 2% FBS, as opposed to 10% FBS in the growth medium. The cells were seeded at 500,000 cells/ml and grown overnight in culture flasks (surface area: 25 cm2) at 37 °C and 5% CO_2 until a monolayer was formed.

The cell cultures were infected with Phelps or Fontes strains and grown at 37 °C and 5% CO_2 until 80–90% of the monolayer was destroyed. The flasks were frozen at –20 °C; after thawing, adenovirus titers were determined as described below.

The cells were plated in 24-well plates at 500,000 cells/ml and cultured overnight at 37 °C and 5% CO_2 until a monolayer was formed. Then, the cells were infected with serial tenfold dilutions (from 10^{-1} to 10^{-7}) of the viral stocks and incubated for 30 min at room temperature. After that, the cells were washed in culture medium; MEM was mixed with Avicel (SigmaAldrich; USA) at a 1 : 1 ratio and added to the washed cells. Then, the cells were incubated for 96 h at 37 °C and 5% CO_2. After that, the cells were stained with 1 ml of 0.1% alcohol crystal violet for 15 min, washed with distilled water, dried at room temperature, and viral plaques were counted in each well. Based on the obtained counts, viral titers were determined using a method proposed by Reed and Muench [12]; the titers were expressed as PFU/ml.

Virus-containing allantoic fluid

Influenza virus was cultured in 9–11-day old embryonated chicken eggs. The embryos were challenged with 102,0–104,5 EID_{50}/0.2 ml. The eggs inoculated with type A influenza virus were incubated at 35 °C for 48 h; those infected with type B influenza virus were incubated for 72 h. After incubation, the eggs were cooled and the virus-containing allantoic fluid (AF) was harvested.

Viral concentrates (VC)

AF was filtered through a cascade of 10, 6 and 1 μm filters and run through a 300 kDa ultrafiltration unit. The obtained concentrate was centrifuged in a sucrose density gradient (60–20%). Then, 40–25% gradient fractions were collected.

Statistical analysis

Statistical analysis was conducted in Microsoft Excel 365 (Microsoft corp.; USA) and Minitab 19 (Minitab Inc.; USA) and involved calculation of 95% confidence intervals.

RESULTS

Optimum cell line for avian adenovirus production

Three candidate cell lines were tested: Vero, MA-104 and HEp-2. These are the most commonly used cell lines for the propagation of adenoviruses. The infectious titers of CELO and Fontes viruses cultured in these cell lines are provided in Table 1.

Both adenoviruses propagated in Vero cells more effectively than in MA-104 and HEp-2: their titers in Vero cells were by at

Table 1. Infectious titers of CELO and Fontes adenoviruses cultured in different cell lines

Virus	Infectious titers, PFU/ml		
	Vero	HEp-2	MA-104
CELO	$4.3 \pm 2.3 \times 10^6$	$6.5 \pm 3.0 \times 10^3$	$1.1 \pm 0.2 \times 10^3$
Fontes	$3.3 \pm 1.5 \times 10^6$	$9.7 \pm 3.8 \times 10^3$	$2.0 \pm 0.7 \times 10^3$

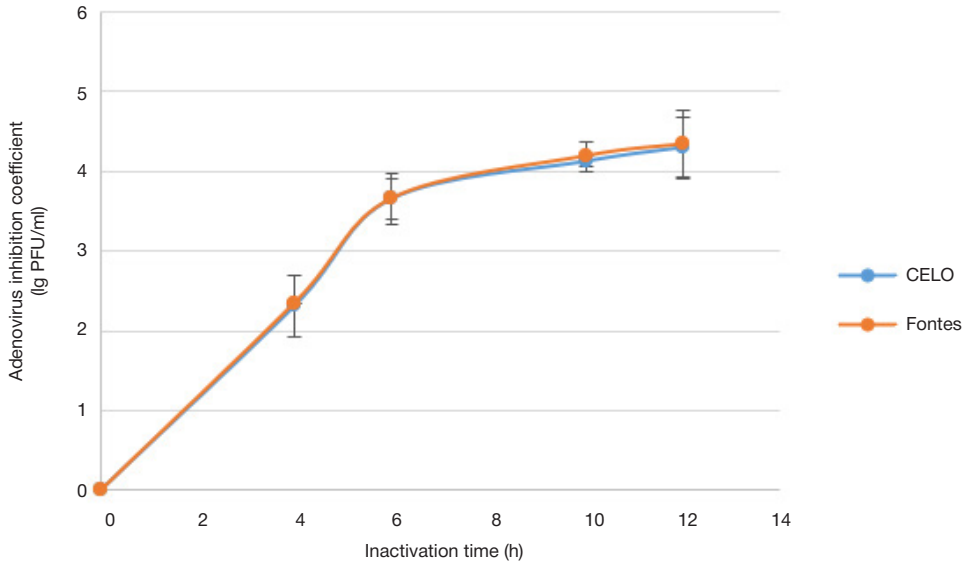

Fig. 1. Dynamics of CELO and Fontes inactivation in the presence of β-propiolactone

least 2 lg higher. In other words, Vero cells turned out to be the most permissive cells for both studied avian adenoviruses.

Dynamics of avian adenovirus inactivation in allantoic fluid by β-propiolactone

To model inactivation of avian adenoviruses in allantoic fluid by β-propiolactone, the titrated viral stock (10% of the AF volume) was added to AF so that the final viral titer was at least 105 PFU/ml. The mixture was inactivated with β-propiolactone (0.09% in the final mixture) and viral titers were measured in the samples. Inactivation dynamics are shown in Fig. 1.

A reduction in viral titers by at least 4 lg PFU/ml occurred no sooner than 10 h after adding β-propiolactone; in other words, allantoic fluid used in the production of influenza vaccines should be exposed to β-propiolactone for inactivation for at least 10 h.

Dynamics of avian adenovirus inactivation in virus concentrates by exposure to UV light

To model inactivation of avian adenoviruses in VC by irradiation with UV light, the titrated viral stock (10% of the VC volume) was added to VC so that the final viral titer was at least 10^5 PFU/ml. Contaminated VC was placed in 90 mm Petri dishes. The dishes were exposed to 4 UV lamps (total power: 60 W) installed at a 20 cm distance from the dishes. The following UV irradiation protocol was applied:

Dish 1 — 0 s;
Dish 2 — 30 s;
Dish 3 — 1 min;
Dish 4 — 2 min;
Dish 5 — 5 min.

After time points specified in the protocol, 1ml samples of VC were collected from the dishes to quantify the number of plaques and thus determine the viral titer. Inactivation dynamics are shown in Fig. 2

A reduction in viral titers by at least 4 lg PFU/ml occurred no sooner than after 5 min of exposure; in other words, exposure to UV light for the inactivation of viral particles in allantoic fluid during the production of influenza vaccines should last at least 5 min.

Dynamics of avian adenovirus inactivation in virus concentrates by detergents

To model inactivation of avian adenoviruses in VC by exposure to detergents, the titrated viral stock (10% of the VC volume)

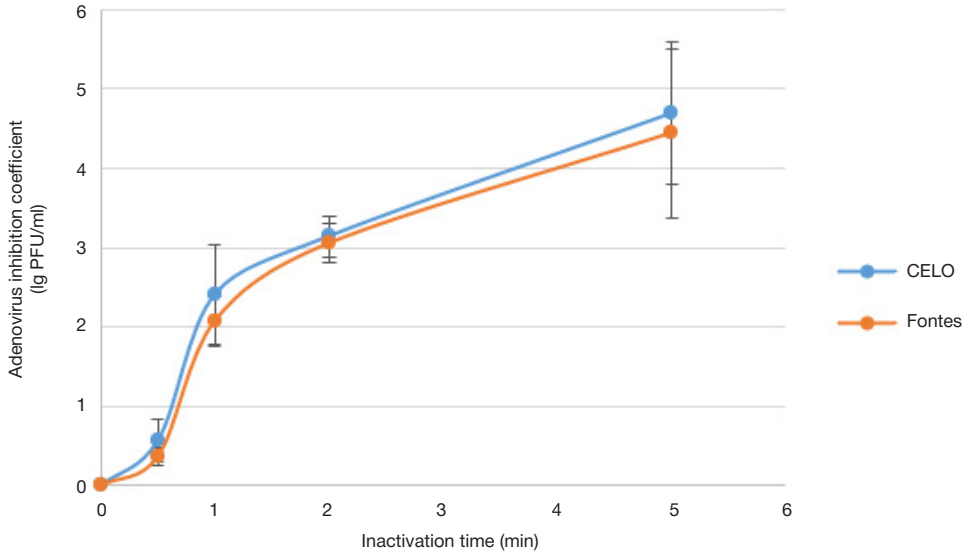

Fig. 2. Dynamics of CELO and Fontes inactivation by UV light

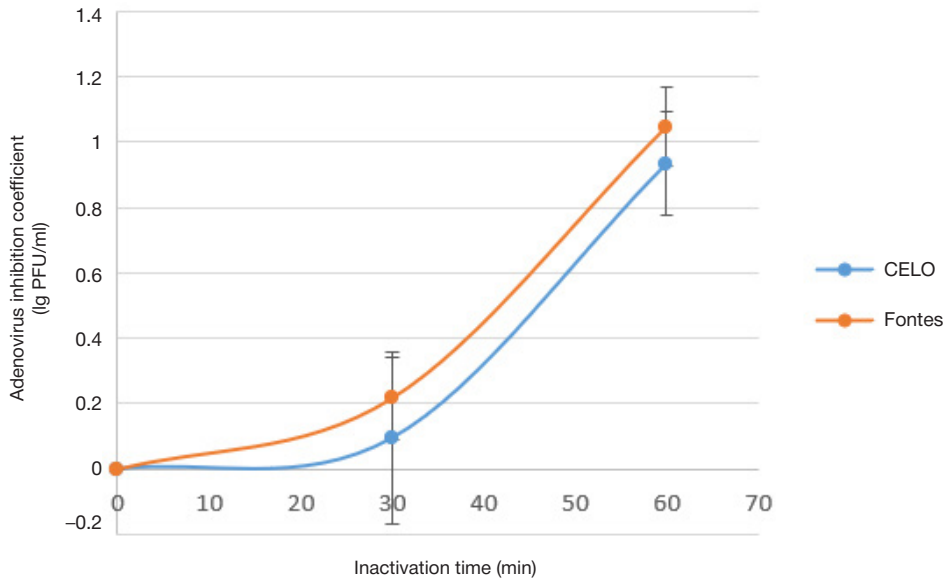

Fig. 3. Dynamics of CELO and Fontes inactivation by n-octyl-β-D-glucopyranoside

was added to VC so that the final viral titer was at least 10^5 PFU/ml. Then, contaminated VC samples were combined with the solutions of n-octyl-β-D-glucopyranoside (total protein to detergent ratio: 1 : 8) or tetradecyltrimethylammonium bromide (total protein to detergent ratio: 1 : 0.5) in PBS, and viral titers were determined. Inactivation dynamics are shown in Fig. 3 and 4.

A reduction in viral titers by at least 1 lg PFU/ml occurred after 1 h of exposure to the detergents. Following exposure to n-octyl-β-D-glucopyranoside, CELO titers fell by 0.93 ± 0.15 lg and Fontes titers fell by 1.04 ± 0.12 lg. With tetradecyltrimethylammonium bromide, CELO titers fell by 1.18 ± 0.17 lg and Fontes titers fell by 1.12 ± 0.38 lg.

DISCUSSION

Inactivation by β-propiolactone and by exposure to UV light is effective against the avian adenovirus strains Fontes and CELO. However, the variability of the results is greater for UV irradiation (Table 2).

These findings may indicate that the UV-based inactivation method is less reliable and may increase the risk of producing a poor-quality influenza vaccine. Most pharmaceutical companies in Russia and abroad employ chemical methods of inactivation. For example, Novartis, ID Biomedical Corp of Quebec and Saint Petersburg Research Institute of Vaccines and Serums (FMBA, Russia) use β-propiolactone as an inactivating agent in the production of influenza vaccines [13–15]. Apart from influenza virus, β-propiolactone can inactivate avian adenoviruses, which are potential contaminants of influenza vaccine intermediates.

CONCLUSIONS

We have selected the optimum cell line for the propagation of Fontes and CELO adenoviruses: Vero cells allow more effective propagation (~ by 2 lg) of these adenovirus strains than Hep-2 and MA-104 cells. Virus-containing allantoic fluid used in the production of influenza vaccines should be exposed to β-propiolactone for inactivation for at least 10 h to ensure a reduction in avian adenovirus titers by 4 lg PFU/ml. If inactivation is performed with UV light, exposure should last at least 5 min to reduce viral titers by 4 lg PFU/ml. In the production of split influenza vaccines, an additional reduction in viral titers by 1 lg PFU/ml can be achieved by using detergents.

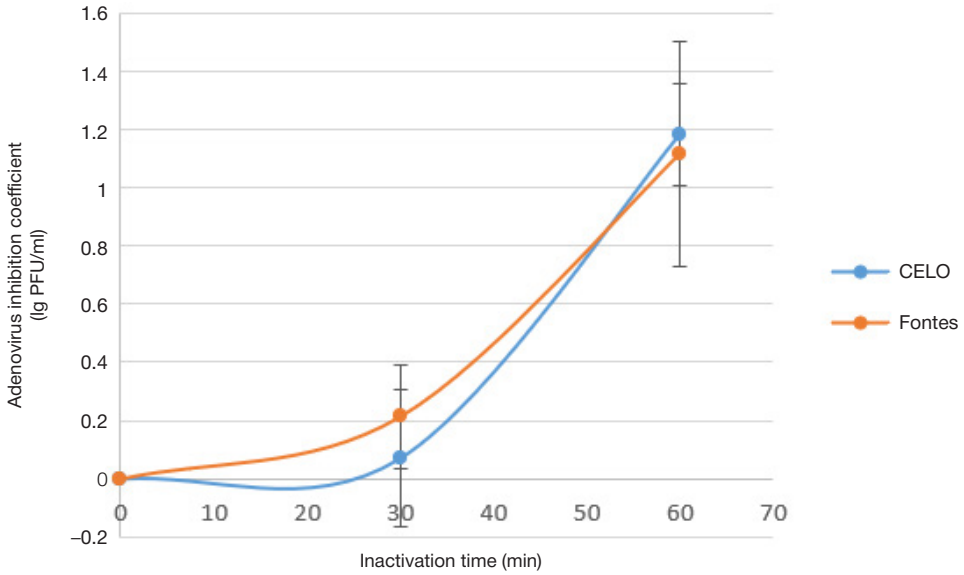

Fig. 4. Dynamics of CELO and Fontes inactivation by tetradecyltrimethylammonium bromide

Table 2. A reduction in adenovirus titers following exposure to different inactivating agents

Inactivating agent	Strain	
	CELO	Fontes
β-propiolactone (inactivation time: 10 h)	4.12 ± 0,06 lg	4.20 ± 0.19 lg
UV light (inactivation time: 5 min)	4.69 ± 0,89 lg	4.44 ± 1.06

So, the technology of influenza vaccine production that involves inactivation of allantoic fluid by β-propiolactone for 10 h followed by inactivation with detergents for 1 h guarantees complete inactivation of avian adenoviruses in the vaccine. However, avian adenoviruses are not the only vaccincontaminants, and further research is needed to study the kinetics of β-propiolactone-based inactivation of avian leukosis virus and mycoplasmas.

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