J Ind Microbiol Biotechnol (2013) 40:625–632
DOI 10.1007/s10295-013-1256-8

FERMENTATION, CELL CULTURE AND BIOENGINEERING

Production of avian influenza virus vaccine using primary cell cultures generated from host organs

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Received: 4 January 2013 / Accepted: 26 February 2013 / Published online: 21 March 2013

Abstract The global availability of a therapeutically effective influenza virus vaccine during a pandemic remains a major challenge for the biopharmaceutical industry. Long production time, coupled with decreased supply of embryonated chicken eggs (ECE), significantly affects the conventional vaccine production. Transformed cell lines have attained regulatory approvals for vaccine production. Based on the fact that the avian influenza virus would infect the cells derived from its natural host, the viral growth characteristics were studied on chicken embryo-derived primary cell cultures. The viral propagation was determined on avian origin primary cell cultures, transformed mammalian cell lines, and in ECE. A comparison was made between these systems by utilizing various cell culture-based assays. In-vitro substrate susceptibility and viral infection characteristics were evaluated by performing hemagglutination assay (HA), 50 % tissue culture infectious dose ($TCID_{50}$) and monitoring of cytopathic effects (CPE) caused by the virus. The primary cell culture developed from chicken embryos showed stable growth characteristics with no contamination. HA, $TCID_{50}$, and CPE exhibited that these cell systems were permissive to viral infection, yielding 2–10 times higher viral titer as compared to mammalian cell lines. Though the viral output from the ECE was equivalent to the chicken cell culture, the time period for achieving it was decreased to half. Some of the prerequisites of inactivated influenza virus vaccine production include generation of higher vial titer, independence from exogenous sources, and decrease in the production time lines. Based on the tests, it can be concluded that chicken embryo primary cell culture addresses these issues and can serve as a potential alternative for influenza virus vaccine production.

Keywords Influenza vaccine production · Primary cell culture · Influenza vaccine timeline

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Introduction

Vaccines, like other biopharmaceuticals, are produced in living systems. An ideal vaccine should be adequately potent and capable of evoking a preventive immune response. Moreover, necessary formulation and manufacturing requirements should be fulfilled that ensure the production of safe vaccine with high production turnover [16]. All the vaccines are dependent upon in vivo processing during a certain phase of production cycle. The reliable and continuous availability of the vaccine production platform is, hence, a major concern for the biopharmaceutical industry [2, 14, 29].

A number of types of vaccines, including live attenuated, inactivated, sub unit and conjugate vaccines, have proved their effectiveness against various pathogenic agents. After attaining the regulatory approvals, these vaccines are now in industrial-scale production [10]. Cell cultures form an essential component of any vaccine production process especially in case of viral vaccines. Attributable mainly to the obligate intracellular nature of viruses and their successful propagation in cell lines, these cell cultures offer adequate vaccine production platforms.

Of the many substrates, embryonated chicken eggs (ECE) have proven to be the most efficient platform for the development of many virus vaccines. Millions of doses are generated annually against susceptible viral pathogens using chicken eggs [23].

Influenza viruses are the most common viral cause of respiratory illness. Its infection is exhibited in both seasonal and pandemic forms. Where the seasonal forms of the virus are responsible for exacerbating the respiratory and cardiovascular conditions in susceptible individuals, the pandemic influenza virus infections cause mortality in nearly 50 % of the reported cases [27]. This high rate of morbidity and mortality is accountable mainly to the therapeutic failure of currently available antivirals. At the molecular level, the influenza virus has an RNA genome that makes it prone to a high rate of genetic mutation. To the benefit of the virus, these mutations can help it in resisting the attack of many antiviral therapies available to date. Vaccination against the virus therefore appears to be the most effective means of curbing this viral pathogen. The currently available influenza virus vaccine has been produced in embryonated chicken eggs since the 1950s [3]. It usually contains hemagglutinin, the chief immunogen of the virus, from the most prevalent forms of human influenza virus. An adjuvant accompanies the viral proteinaceous material in this oil in water emulsion to increase the immune presentation of the antigen [9, 13]. After the inoculation of the virus in the eggs for a particular time period, the eggs are harvested and the virus is chemically inactivated. This egg-based product effectively meets the annual requirement of the vaccine. However, in case of a pandemic, the increased demand of the vaccine and the decreased availability of eggs leads to the failure in its provision to the masses [18]. Therefore, in order to manage the vaccine needs of the global health care system, alternatives need to be found and developed. Cell lines fulfill the criteria of an efficient vaccine production platform. The virus has been found to efficiently propagate on a number of cell lines [15, 17, 26]. The capability of the cell lines to be sub-cultured and passaged makes them available for use in successive production cycles, resulting in the independence on exogenous supplies. Moreover, a decrease in the overall production time is observed. These advantages have therefore helped in attaining the regulatory approval for the use of cell lines for influenza virus vaccine production [1, 6].

A number of factors, however, affect the successful propagation of influenza virus on a particular cell line. The distribution of sialic acid receptors, tissue tropism capability, and the cellular environment are considered the main players responsible for the preferential binding and propagation of the virus for these cell lines [24]. The current study hypothesized that a higher viral titer and a greater antigen yield could be obtained if the virus was made to infect the cell lines derived from their natural hosts. A low pathogenic avian influenza virus strain (H9N2) was selected and introduced into a variety of platforms of both avian and non-avian origin. The overall yield of the virus was compared and the time required for the production of vaccine batches from these sources was determined.

Materials and methods

Avian influenza virus

Field isolates of AIV H9N2 (A/chicken/Pakistan/NCVI-01/2010 (H9N2) were obtained from Poultry Research Institute, Rawalpindi, Pakistan. They were amplified by one additional allantoic passage in chicken embryo to make working stocks of the virus. The infectivity titer of the virus was in the order 10^9 EID_{50}. All experimental procedures involving live AIV H9N2 were performed according to the standard operating procedures of the approved biosafety level-3 facility.

Cell cultures

Cell culture medium

The wash solution used during tissue isolation procedure contained Hanks' balanced salt solution (HBSS) adjusted to pH 7.4 with 4 % w/v sodium bicarbonate solution. The antibiotic stock solution, comprising of 200 units of penicillin, 200 μg of streptomycin, and 100 units of amphotericin B, was added per ml of the medium. Cells were grown in Dulbecco's modified Eagle's medium (DMEM) (D-5648, Sigma Aldrich, USA) supplemented with 10 % v/v fetal bovine serum. This growth medium contained 100 units of penicillin and 100 μg of streptomycin per ml. For the maintenance of all the cell cultures, DMEM containing 2 % v/v fetal bovine serum, 25 units of penicillin, and 25 μg of streptomycin per milliliter was used. All the culture media used were adjusted to pH 7.4 with 4 % w/v sodium hydroxide solution. All other chemicals used in the experiments were of cell culture or analytical grade.

Chicken embryo tissue isolation

Primary chicken cells were prepared from SPF-quality (specific pathogen-free) eggs obtained from Poultry Research Institute, Rawalpindi, Pakistan. The eggs were incubated in an incubator at 38 °C/60 % relative humidity for 9–12 days. Chicken lungs and skeletal muscles were aseptically removed, minced with cross-scalpel technique, and dissociated into single-cell population by immersing in

a 0.25 % trypsin-1 mM EDTA solution at 4 °C overnight. The fluid was removed and cells were pelleted at 1,000 rpm for 10 min. The pellet was re-suspended in the growth media and seeded in 25-cm^2 tissue culture flasks. These flasks were incubated at 37 °C in 4.5 % CO_2 atmosphere (MCO-17A, CO_2 Incubator, Sanyo, UK). Confluent cell monolayers were obtained within 4–5 days. The cells were sub-passaged and plated at 2×10^6 cells per 10 in 25-cm^2 tissue culture flasks. The cell plating density was determined by using the Countess Automated Cell Counter (Invitrogen, USA). Starting from the first sub-culturing of the cells, passage numbers were assigned to each generation. After 17 passages, the lung and skeletal muscle cells were used for viral inoculation.

Transformed cell lines

Three human-origin cell lines were selected for the study in order to compare the infectivity of the virus in non-avian origin cell cultures. Human cervical cancer and hepatoma cell lines were obtained from ATCC and grown in the cell culture medium discussed in the section "Cell culture medium".

Viral inoculation of cell lines

Five human and avian origin cell lines (Table 1) were prepared in 6-well plates at a seeding density of 2×10^6 cells per 10 cm^2 of growth surface. All the cell lines were inoculated at a multiplicity of infection (M.O.I.) of 1 with AIV H9N2 strain. In order to ensure the adherence of the virus to the cell cultures, the plates were incubated for 2 h along with the addition of 1 μg of trypsin–EDTA per milliliter of the medium. Non-attached virus was removed by washing the cells twice with serum-free DMEM. All infected cell lines were incubated at 37 °C for 5 days in a 5 % CO_2 incubator. Cytopathic effects (CPE) were observed by inverted-phase contrast microscopy at days 1, 3, and 5 (TCM-400, OEM-Optical, Labomed, USA).

Table 1 The human and avian origin cell lines used in the study

Type of cell line	Name	Source of cell line
Human		
Cervical adenocarcinoma	HeLa	ATCC no.- CCL-2
Hepatoma	Huh	NCVI cell bank
Hepatoma	Hep G	NCVI cell bank
Avian		
Chicken embryo lung	CEL	In-house development
Chicken embryo skeletal muscles	CEM	In-house development

Viral inoculation of embryonated chicken eggs

The stock virus was also inoculated into embryonated chicken eggs. A total of 100 μl of viral stock was injected into the allantoic fluid of the egg and then the eggs were incubated at 38 °C for 5 days. They were rotated at 180° in the horizontal plane twice daily. On the completion of the incubation period, the eggs were killed by refrigerating overnight. The allantoic fluid was collected and processed for viral titer assays.

Evaluation of viral propagation on various platforms

AIV titers were determined by cytopathic effects (CPE) scoring, 50 % of tissue culture infectious dose ($TCID_{50}$ per ml) and hemagglutination assay (HA). For CPE analysis, after draining the growth medium from the confluent monolayers of cells, 1 ml of 0.5 μg/ml Trypsin was added prior to the introduction of the virus and incubation into the cell lines. After the incubation period, the cells were observed for rounding, disaggregation, erosion from the flask base, and/or vacuolization in the cell layers. The absence of any CPE was marked as '0' while complete deformation was assigned a score of 4. In case less than one-fourth of the monolayer was affected, a score of '1' was given, less than half but greater than one-fourth got '2', while a score of '3' was assigned to the cell cultures showing 50–75 % deformation. To perform the TCID50 assay, the chicken embryo lung cell line in 96-well plates was inoculated with tenfold serial dilution of virus stocks isolated from the supernatant of various cell lines with trypsin and incubated at 37 °C, in 5 % CO_2; 50 % of tissue culture infective doses (TCID50/ml) were examined on days 1, 3, and 5 post-infection (d.p.i.). The HA titer of the virus was determined as the reciprocal of the highest suspension that showed complete agglutination of chicken RBCs. The test provided the approximate number of agglutinating virus particles per ml of the supernatant obtained from various cell lines. The test sample to control ratio throughout the study was kept at 1:1. The results were processed using the Statistical Package for Social Sciences, (SPSS v 16.0 for Windows, SPSS Inc., Chicago, IL, USA) at a $p < 0.05$.

Production batch cycle time

A comparison was made between the time required for the attainment of a potent end-product using the egg-based and cell culture-based platforms. For the purpose, time period was noted from the attainment of raw material until the achievement of characterizable antigen titer.

Results

Establishment of chicken embryo primary cell culture

Cells were isolated from various organs of 9- to 2-day-old chicken embryos and were propagated in tissue culture flasks. These cells originated from cardiac, respiratory, muscular, and gastro intestinal tissue. Under the growth conditions mentioned, only chicken embryo lung and skeletal muscles showed signs of healthy propagation. They started forming foci within a few days of plating and it took approximately 5–7 days for the cells to form a confluent monolayer of cells. Sub-passaging was done during the whole course of the study (33 weeks) and the chicken embryo lung and chicken embryo skeletal muscles showed varying yet healthy growth patterns.

Infection ability of the virus on various cell lines

Avian influenza virus was inoculated onto the various cell cultures in order to compare the susceptibility to these platforms. Hemagglutination assay (HA) was performed on the supernatant isolated from cell cultures and the embryonated chicken eggs. HA titer and agglutinating viral particles were determined after 5 days p.i. (Fig. 1). It was observed that the viral titer obtained from the chicken embryo primary cell cultures was comparable with the one obtained from the embryonated chicken eggs. However, the human origin cell lines had considerably lower (approx. tenfold) viral titer. The highest titer in the tested non-avian cell lines was observed in the human cervical cancer cell line (HeLa). The human hepatoma cell lines did not produce any significant rise in the viral titers.

For the quantification of live virus obtained from the suspensions of cell cultures and embryonated chicken eggs, the TCID $_{50}$/0.1 ml was determined on the 5th day following the viral infection. The highest titer of the virus per 0.1 ml of the suspension was obtained from the allantoic fluid of the ECE. Among the tested cell lines, the supernatants obtained from infected chicken embryo cell lines, though considerably lower than the ECE suspension, had the maximum viral titer (Fig. 2). There was a difference of up to 3.5 $\log_{10}$ between the TCID $_{50}$/0.1 ml values of the chicken embryo cell lines and the human origin cell lines, showing the non-permissive nature of the human cell lines for AIV growth.

The virus tends to affect the morphology and growth patterns of the cells upon infection. Characteristic CPE were observed in the cell lines after their inoculation with AIV. CPE scoring was performed under inverted-phase contrast microscopy at days 1, 3, and 5 post-infection (Fig. 3). The AIV produced limited CPE in the human origin cell lines (HeLa, Huh, and Hep G). In comparison, the chicken embryo lung and muscle cell cultures were found to be much more susceptible to AIV infection, as around 75 % of the cell monolayer was destroyed by the 5th day of the assay. This confirmed the higher preference of the AIV for the chicken embryo cell systems and, hence, a higher CPE score was observed for these cells (Fig. 4). Furthermore, to confirm that AIV as the causative agent of the CPE observed, AIV matrix gene was amplified and

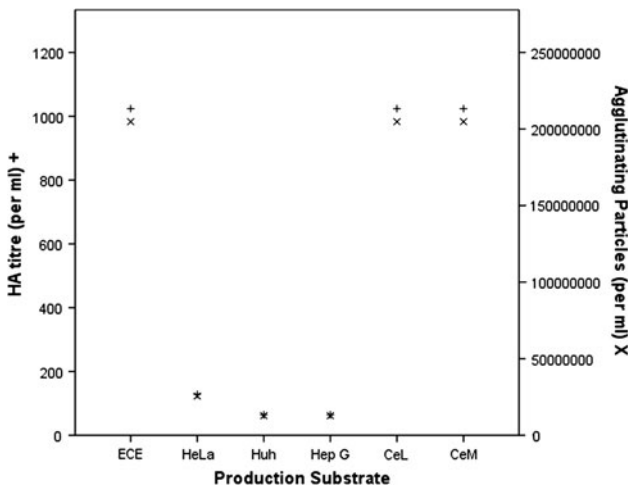

Fig. 1 Comparison of the hemagglutinin titer and the agglutinating viral particles per milliliter of the supernatant obtained from various cell lines (HeLa, Huh 7, Hep G, CeL, and CeM) and embryonated chicken eggs on infection with AIV H9N2 strain

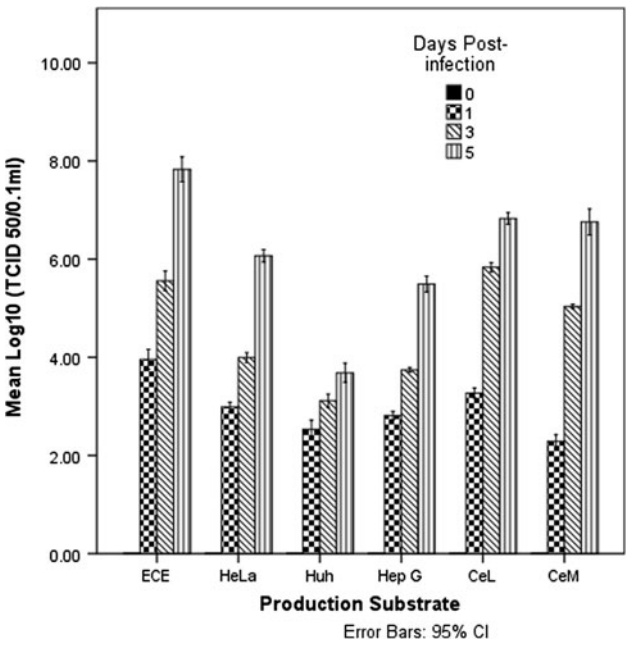

Fig. 2 Representation of viral replication patterns by the 50 % tissue culture infectious dose (log $TCID_{50}$/0.1 ml) assay of AIV (H9N2) obtained from human (HeLa, Huh 7, and Hep G) and avian origin cell lines (CeL and CeM) in comparison with the ECE

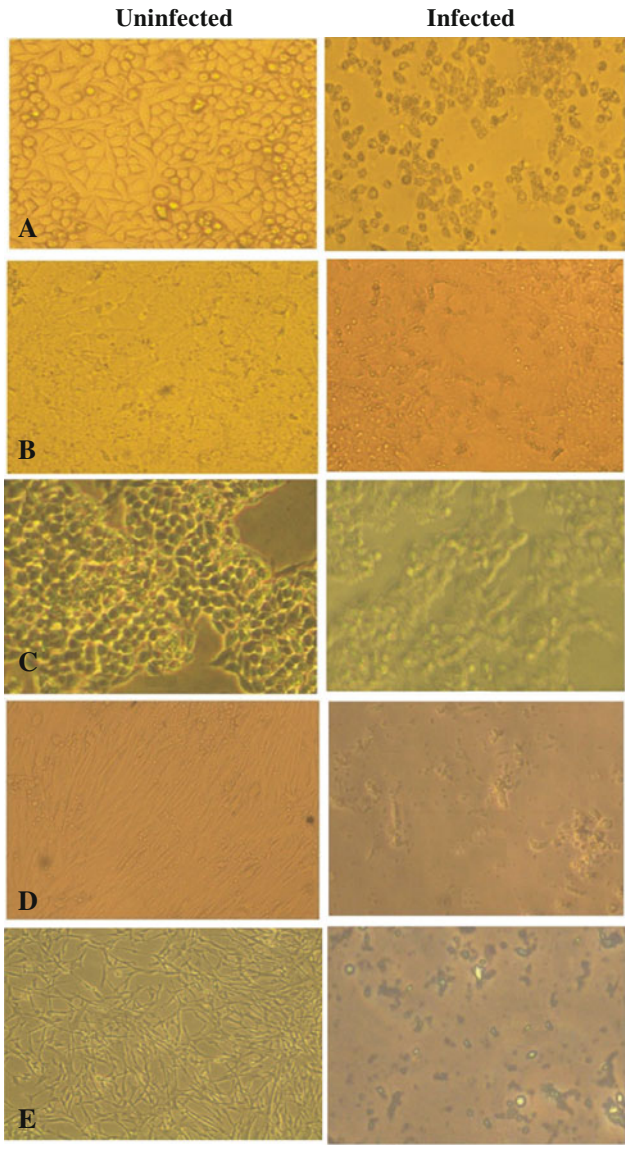

Fig. 3 The degree of cytopathic effects observed in various cell systems; uninfected cell lines (*left column*) versus the cell lines infected with avian influenza virus (H9N2) (*right panel*). The cell lines are **a** human cervical cancer cell line, HeLa, **b** human hepatoma cell line, Huh 7, **c** human hepatoma cell line, Hep G2, **d** chicken embryo lung primary culture, CeL and **e** chicken embryo skeletal muscle primary culture, CeM

sequenced. Strong sequence homology confirmed the infection of the cell lines by AIV H9N2 strain. The consensus sequence was reported to GenBank (Accession Number JN200413).

Batch completion time

The time required for the production of a batch of vaccine from the cell culture and embryonated chicken eggs was compared (Fig. 5). On acquiring the freshly laid embryonated chicken eggs, 8 ± 1 days were required before the

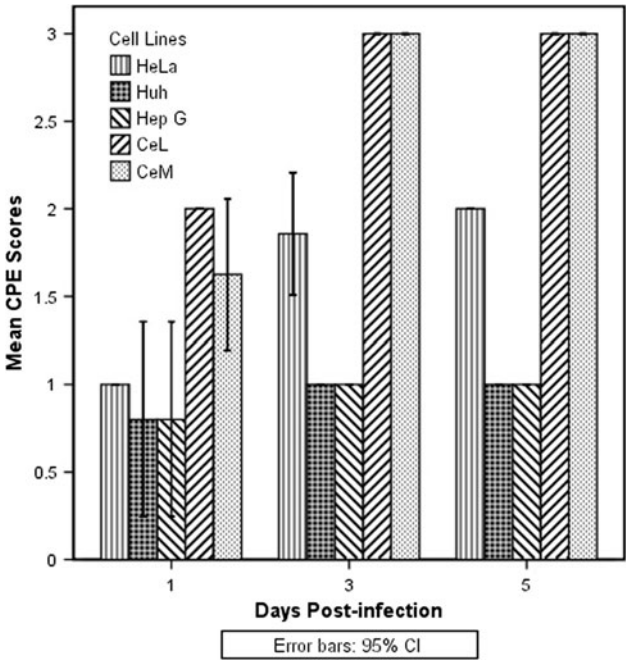

Fig. 4 Mean cytopathic effect (CPE) scores of various cell lines (HeLa, Huh 7, Hep G, CeL, and CeM) infected with avian influenza virus H9N2 (A/chicken/Pakistan/NCVI-01/2010 (H9N2) at days 1, 3, and 5 post-infection

virus could be introduced into the egg for maximum viral titer. An additional 6 ± 1 days were needed for the propagation of the virus in the egg. However, an initial period of 3.5 ± 0.5 days was required for the cell line-dependent procedure to develop confluent monolayers. It is followed by 4 ± 0.5 days for effective viral infection. Moreover, the overall suspension attained from the ECE was 11 ± 2 ml per egg. However, ten times-greater viral suspension was attained from the cell lines infected with the virus.

Discussion

The provision of effective influenza virus vaccine to the general population remains an uphill task for the public health authorities around the world. This is especially observable during the influenza pandemic period. The recent pandemics have resulted in severe disease in a broader spectrum of hosts, both animals and humans. It can be attributed mainly to an increased pathogenicity and wider geographical distribution of the virus [11, 19, 21]. The currently licensed process for vaccine manufacturing relies greatly on the provision of embryonated chicken eggs. Consequently, during a pandemic period, an increase in the death of chicken is coupled with a decreased supply of eggs. This, in turn, leads to the in-availability of the influenza virus vaccine to the susceptible populations. Alternate methods are under development for the

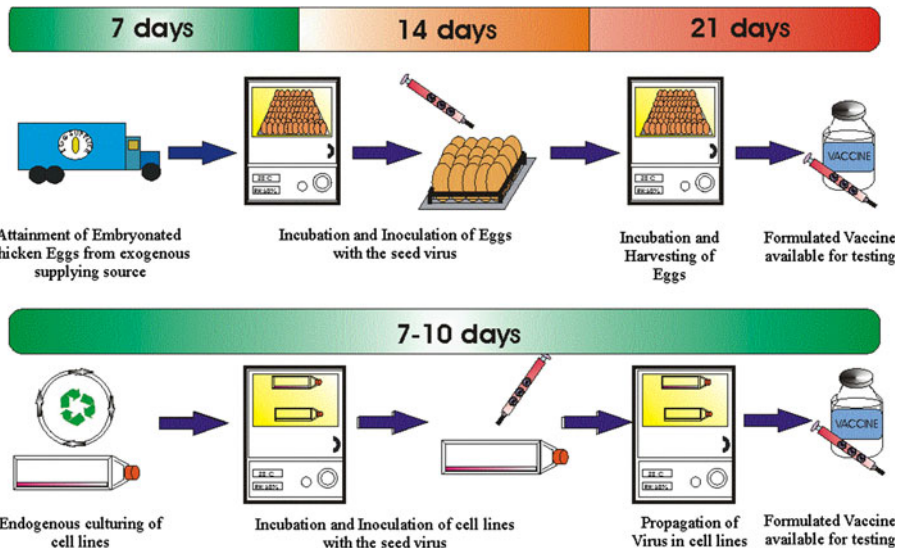

Fig. 5 Timeline for the production of influenza virus experimental vaccine using the embryonated chicken eggs (*upper panel*) and primary cell culture technique (*lower panel*)

production of effective vaccine. The use of cell culture has been studied earlier, which suggests the adoption of this system for industrial-scale production. However, most of the research groups have focused on the use of mammalian cell systems for the vaccine production of all the influenza viruses, whether of human, avian, or swine origin [5, 15, 17, 26]. The development of primary cell cultures derived from chicken embryos can provide an excellent platform for the purpose. Phylogenetic models have revealed a close association of all influenza viruses with birds [12]. Therefore, selecting an avian cell culture for the propagation of AIV can serve as an excellent alternative for the currently recommended cell lines. Moreover, primary cell cultures closely resemble the in vivo environment as compared to the transformed cell lines [7]. The current study was therefore designed to determine the use of avian-origin primary cell cultures for their capability to produce influenza vaccine. Moreover, a comparison with the conventional embryonated chicken egg production pathway was made.

Chicken embryos were used to develop avian origin primary cell cultures for studying viral infection. These cell lines can, subsequently, be used as a platform for vaccine production. Readily available raw materials were used in the process. Though the population doubling times varied, the growth patterns were constant, and a confluent monolayer was obtained on the growth surface within 4–5 days. After subsequent passaging, certain cell systems did enter into senescence crisis, between sub-passage number 10 and 15. These cells were removed and the viable cells were used for further experimentation. Overall, the primary cell culture-derived cell line was effectively maintained and stable growth was observed until the completion of the study. Since no tool for introduction of tumorigenesis was introduced; therefore, a major safety concern becomes invalid, which is objected in case transformed cell lines are used [4].

An important requirement for a successful vaccine manufacturing process is the effective infectivity of the inoculum in the production platform. The differential susceptibility of the virus is indicative of molecular and cellular predisposition for the different systems [20, 24, 25]. The study systematically compared the permissibility of various cell lines for the avian influenza virus and related them with the production efficacy against the licensed embryonated chicken eggs system. Hemagglutination assay, exhibiting the antigen generation capability, showed that all the cell lines were able to raise the viral titer to a certain extent. The avian influenza virus showed maximum preference for the cell cultures developed from chicken

Table 2 The hemagglutinin assay, agglutinating particles, cytopathic effects, and 50 % tissue culture infectious dose results observed on the 5th day of assay

Cell systems	Hemagglutinin assay (per ml)	Agglutinating particles (per ml)	Cytopathic effects	$TCID_{50}$ (per 0.1 ml)
Embryonated chicken eggs	1,024	2.048×10^8	–	8.0
HeLa	128	2.56×10^7	2	6.0
Huh 7.5	64	1.28×10^7	1	4.0
Hep G	64	1.28×10^7	1	5.7
CEL	1,024	2.048×10^8	3	6.9
CEM	1,024	2.048×10^8	3	6.9

embryos. The hemagglutinating particles thus produced were very close to those produced in ECEs. Human origin cell lines, however, failed to prove their effectiveness to raise the viral titer, significantly. The maximum HA-based viral titer, among the non-avian cell lines, was observed in the human cervical cancer cell line, HeLa. The viral growth capability of human origin virus on HeLa has been investigated earlier and similar results have been obtained. The human hepatoma cell lines minimally supported the viral growth. However, it was observed on the basis of hemagglutination assay that the avian influenza virus has a strong preference for the growth in avian origin cell lines as opposed to the secondary hosts.

Complementing the HA assay, the 50 % tissue culture infectious dose assay presented similar results. The assay, which is indicative of the viral replication in the cell systems, demonstrated that the maximum viral load per milliliter of the final suspension was obtained from the embryonated chicken eggs. Among the cell systems, chicken embryo lung and muscle cells gave higher viral titers, almost 3.5 times greater than the human origin cell lines. However, the overall cell suspension obtained from the cell culture techniques was nearly ten times greater than the egg suspension. This indicates a greater viral and, hence, vaccine output. The cell suspension attained from an embryonated chicken egg under optimized conditions contains hemagglutinin units, which are sufficient for only one vaccine dose or even lesser [22]. The cell cultures present a feasible option for influenza vaccine production, which is based on the greater volume and viral titer attained from the cell suspension. In the last step of testing the preferential affinity of the virus for the cells under study, the cytopathic effects exhibited by the virus were observed. Most prominent CPE was observed in the chicken embryo lung and muscle cell lines, which correlated with the presentation of severe symptoms in the avian population [8]. Human cell lines did not very effectively produce the CPE, except for the human cervical cancer cell lines.

The tests exhibited a high permissibility of the avian influenza virus for avian origin cell systems giving a higher antigen yield, viral load, and cell infectivity (Table 2). The growth capability of the human reassortant of the virus on the mammalian cell lines has been studied in a number of projects earlier and notable benefits have been observed in comparison to the ECEs [15, 17, 26, 28]. Our study further strengthens the conclusions made earlier that human influenza viruses can propagate more efficiently on the human origin cell lines. Similarly, avian cell systems can serve as a better substrate for the avian influenza virus. Lastly, the time for the production of the end product from the two routes, i.e., cell system-based and embryonated chicken eggs-based, was compared. It was observed that the cell lines-based protocol required nearly half the time than that required for the ECE-based vaccine. Moreover, the dependence on supplier was eliminated, making the procedure highly self-reliant and easy to validate.

Data from the study suggests that the host origin primary cell cultures can serve as a valuable tool for the industrial production of influenza virus vaccine. Being highly permissive to viral growth, this system could be of great industrial interest. Although process optimization and scale-up studies are necessary, the developed protocol can help to reduce the industrial dependence upon the exogenous viable supplies, decrease the production time line, and increase the overall vaccine output.

Acknowledgments The work was supported by National University of Sciences and Technology, Pakistan. The Avian Influenza Virus H9N2 was obtained from the Poultry Research Institute, Rawalpindi. We are grateful to Dr. Florian Krammer for his technical advice and expertise.

Conflict of interest None.

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