



# Development, characterization and optimization of a new suspension chicken-induced pluripotent cell line for the production of Newcastle disease vaccine



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## ARTICLE INFO

### Article history:

Received 26 March 2015

Received in revised form

11 August 2015

Accepted 15 September 2015

Available online 14 November 2015

### Keywords:

Newcastle disease virus

Vaccine production

Suspension cell culture

Chicken-induced pluripotent cell

Serum free medium

## ABSTRACT

Traditionally, substrates for production of viral poultry vaccines have been embryonated eggs or adherent primary cell cultures. The difficulties and cost involved in scaling up these substrates in cases of increased demand have been a limitation for vaccine production. Here, we assess the ability of a newly developed chicken-induced pluripotent cell line, BA3, to support replication and growth of Newcastle disease virus (NDV) LaSota vaccine strain. The characteristics and growth profile of the cells were also investigated. BA3 cells could grow in suspension in different media to a high density of up to  $7.0 \times 10^6$  cells/mL and showed rapid proliferation with doubling time of 21 h. Upon infection, a high virus titer of  $1.02 \times 10^8$  EID<sub>50</sub>/mL was obtained at 24 h post infection using a multiplicity of infection (MOI) of 5. In addition, the cell line was shown to be free of endogenous and exogenous Avian Leukosis viruses, Reticuloendotheliosis virus, Fowl Adenovirus, Marek's disease virus, and several Mycoplasma species. In conclusion, BA3 cell line is potentially an excellent candidate for vaccine production due to its highly desirable industrially friendly characteristics of growing to high cell density and capability of growth in serum free medium.

Published by Elsevier Ltd on behalf of The International Alliance for Biological Standardization.

## 1. Introduction

Newcastle disease (ND) is a major poultry disease causing significant economic losses and is a major threat to food security in

many countries [1]. It is the most devastating disease for rural poultry, especially in the developing world [2]. The disease has been reported in most bird species with chickens being the most susceptible and the disease may range from mild, with little or no apparent clinical signs, to severe with 100% mortality [3].

Vaccination is the most accepted prevention and control strategy for combating ND in poultry worldwide. Commonly used vaccines are made with low virulence viruses, which provide both cellular and humoral immunity at a low cost, and inactivated oil-emulsion of the same viruses which confer a higher and long lasting humoral immunity [4–6]. In addition different recombinant NDV vaccines based on low virulence avian viruses (Herpesvirus of Turkey, Fowlpox or low virulence NDV) have been developed and

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used with great success experimentally. Recombinant vaccines are generated by cloning the fusion (F) and hemagglutinin-neuraminidase (HN) gene of circulating genotype into a viral backbone this thereby provides protection against clinical disease and shedding of virulent challenge virus [7–10]. Different vaccines are commercially available and live ND vaccines are largely produced in specific pathogen free (SPF) chicken embryonating eggs. The production of SPF eggs requires significant resources for the procurement and maintenance of a disease free rearing facility; hence there is a limited number of facilities globally producing SPF eggs [11]. In times of high demand other vaccine manufacturers (e.g. avian influenza) import SPF eggs, thus creating challenges for availability, logistics, and costs. The high dependence of vaccine production on perishable SPF embryonating eggs, loss of birds to disease, or antibody development in surviving birds are factors that often renders flock and eggs unusable for vaccine production and may stall manufacturing. These limitations are accentuated in the developing world where SPF eggs are expensive and not always available to vaccine producers during outbreaks.

MDCK and Vero cell lines have been extensively studied and used by manufacturers for the production of approved influenza virus vaccine [12–14]. Unlike influenza virus vaccine production, limited avian cell lines have been explored for NDV vaccine production [15,16] hence manufacturing of NDV vaccine largely depends on embryonated eggs for production. Some primary and continuous cell lines of avian and non-avian origin have been used in the propagation of NDV. These include chick embryo fibroblast (CEF), chicken embryo liver (CEL) cells, chicken embryo kidney (CEK) cells, African green monkey kidney (Vero) cells, and DF-1 (a cell line derived from CEF) cells [16,17]. Primary cells have the disadvantage of senescence leading to the need for regular and costly re-derivation and characterization and the fact that they may not always comply with modern quality standards required for good manufacturing practices. These cells are anchorage dependent and not always easily amenable to scale up for large scale vaccine production, which is often done in suspension to maximize space and cell density [18]. Use of suspension culture makes vaccine production easier because there is no need for trypsinization or anchorage support for the cells. This has created significant interest in developing immortal and stable cell lines with the capability of growth in suspension. Additionally, it is also of importance to be able to expand cells in serum free medium for vaccine production as it eliminates a potential contamination (e.g. prion, viral) source and significantly reduces production cost, which is critical as most chicken vaccines must be economical to be cost effective for producers. It is also of significant interest to produce vaccine virus in cells from the target host species (*Gallus gallus*) to prevent mutations and reversion to virulence that may make it less effective as a vaccine.

In recent studies, researchers have succeeded in growing adherent cells like Vero and MDCK on micro-carriers [19,20] or adapting them to grow in suspension [21,22] for Influenza vaccine production. In other attempts, “designer” cell lines like human PER.C6 [23], EB66 [24], AGE1.CR.pIX [25], and CAP [26] with industrial friendly properties have been developed and used for vaccine production. In order to add to the choice of cell lines available for vaccine production a new cell line derived from non-viral minicircle DNA method of reprogramming was developed [27]. This process of cell reprogramming has received the approval of the Food and Drug Administration (FDA) and is being used widely for research and development [28].

This study focuses on describing, for the first time, the characteristics of a newly developed serum-free, suspension chicken-induced pluripotent cell (SciPC) line BA3 and determining the potential of this cell line as an alternative to egg based ND virus

vaccine production. The BA3 cell line was tested and found to be free of endogenous and exogenous avian retroviruses as well as other adventitious agents. This makes it a potential candidate for the production of animal and human vaccines on an industrial scale.

## 2. Materials and methods

### 2.1. Cell line

The BA3 ciPC line was derived as previously described by Lu et al. [29]. Briefly, day 11 chicken embryos were used to isolate for chicken embryonic fibroblasts (CEFs). CEFs were cultured in fibroblast medium (DMEM high glucose (Hyclone) with 10%FBS (Hyclone), 4 mM L-Glutamine (Gibco) and 50 U/mL penicillin and 50 µg/mL streptomycin (Gibco)) in 5% CO<sub>2</sub> at 37 °C, and split using 0.05% trypsin (Gibco) as they reached to confluence. For transduction, a total of 150,000 CEFs were plated in one well of a 12-well plate. After 24 h, CEFs underwent lentiviral transduction utilizing the viPC kit (Thermo Scientific) with viruses containing the human stem cell genes POU5F1, NANOG, SOX2, LIN28, KLF4 and C-MYC under the promoter of human elongation factor-1 alpha (EF1a) (EF1 underwent lentiviral agents). CEFs were trypsinized 24 h after transduction and passaged onto inactivated feeder cells in embryonic stem cell expansion medium (Dulbecco's modified Eagle medium (DMEM)/F12 (Gibco), supplemented with 20% knockout serum replacement (KSR; Gibco), 2 mM L-Glutamine (Gibco), 0.1 mM non-essential amino acids (Gibco), 50 U/mL penicillin, 50 µg/mL streptomycin (Gibco), 0.1 mM mercaptoethanol (Sigma–Aldrich) and 10 ng/mL basic fibroblast growth factor (bFGF; Sigma–Aldrich and R&D System)). ciPC-like cells were manually harvested and plated on Matrigel (BD Biosciences; diluted 1:100 in DMEF/F12) coated dishes in mTeSR1 medium (Stemcell Technologies). ciPC-like cells were mechanically dissociated using a glass Pasteur pipette every 4–5 days. Cells were then transferred into 20% KSR embryonic stem cell expansion medium, while still plated on Matrigel. Cells adjusted to the 20% KSR medium and were passaged using 0.05% trypsin once reaching 85% confluence.

#### 2.1.1. Adaptation of BA3 cell line to serum free medium and suspension growth

A step-wise reduction of the percentage of KSR medium (20–1%) was performed to determine optimum concentration for effective cell growth. The resulting cells, named suspension ciPC (SciPC) BA3 cells, from the reduced KSR treatment were grown in a petri dish on a shaker agitated at 50–100 rpm in 5% CO<sub>2</sub> atmosphere at 37 °C. The bFGF from the medium was equally removed.

### 2.2. Characterization of the suspension ciPC line

#### 2.2.1. Growth profile (saturation density and doubling time)

The SciPC cells were grown in DMEM/F12 with and without 15 mM HEPES supplemented with 5% KSR and seeding density of  $7.0 \times 10^5$  cells/mL with continuous agitation on a shaker at 50–100 rpm in 5% CO<sub>2</sub> atmosphere at 37 °C with medium changes every 24 h. Cell counting was performed using Cellometer Auto T4 (Nexcelom Bioscience LLC, Lawrence, MA) with 0.4% trypan blue.

#### 2.2.2. Testing for the presence of avian leucosis, reticuloendotheliosis viruses, Fowl adenovirus, Marek's disease virus, and mycoplasma

One of the major limitations of establishing new cell lines is the presence of congenitally transmitted as well as endogenous viruses, making them unsuitable for vaccine production. Media samples from the BA3 cell line were inoculated onto chicken embryo

fibroblasts (CEFs) of Avian Disease and Oncology Laboratory (ADOL) line 0, a line lacking all endogenous viruses and resistant to infection with subgroup E ALV (C/E) [30], and on ADOL RFS, a line lacking all endogenous viruses, but susceptible to all subgroups of ALV including subgroup E (C/O) [31]. Inoculated and uninoculated control CEFs were incubated at 37 °C. At seven days post inoculation (PI), Tween 80 (0.2% final concentration) was added to the cells, the plates were freeze-thawed twice, and 100 µL of the resulting lysates was tested for the presence of ALV group-specific antigen (p27) protein by enzyme-linked immunosorbent assay (ELISA) [32]. In addition to using ELISA on cell lysates for detection of p27, DNA from inoculated and uninoculated control ADOL CEFs of line 0 and RFS was also extracted for testing for the presence of ALV and REV by Polymerase Chain Reaction (PCR), as previously described [32,33]. We also assessed potential contamination against other agents using PCR. PCR test was conducted in both cell lysates and supernatant and always included a positive control. PCR tests were conducted for Fowl adenovirus, chicken Anemia virus, Marek's disease and Mycoplasma. Briefly, the following reference strains were used in the present study as positive virus strains: RAV-1 (subgroup A), RAV-2 (subgroup B), Rav-49 (subgroup C), RAV-50 (subgroup D), HPRS-103 (ALV-J), Reticuloendotheliosis virus strain 566 (Rev-566), strain CELO FADV1, Chicken Anemia live virus vaccine (CIAV) (Biomune CO, Lenexa, KS 662105, USA) and RB-1B (USA field isolate vvMDV), SB1 + HVT (Merial select vaccine strain), HVT (Merial Select vaccine strain) and Rispen MDV serotype 1 vaccine strain were used as a positive control. Total cellular DNA or RNA were extracted from 1 mL of virus and samples of  $10^6$  cells using the Roche PCR Template kit for DNA (Cat # 11796828001, Roche applied Science, Germany) or High Pure RNA isolation kit for total RNA (Cat # 118282665001, Roche applied Science, Germany) according to the manufacturer's instructions. The concentration of DNA or RNA was measured by NanoDrop lite Spectrophotometer at 260 nm. Two primers of PCR or RT-PCR primers were designed from CELO FAV1 (Hexon gene Genbank accession No. AC\_000014), the forward and reverse of CIAV PCR or RT-PCR primers were designed from CIAV VP3 gene (Genbank accession No. NC001427\_CuX), MDV PCR amplified with an meq RB1B sense and a meq antisense primer and a set of primers that amplify whole gag gene were designed from rev-566 and used for detection of Rev. All primers location, and expected PCR or RT-PCR amplicon sizes for each virus were shown in [Supplementary Table 1](#). For conventional PCR provirus DNA and extracted DNA segments were amplified using RAV-1, RAV-2, RAV-49, RAV-50 (Subgroup A, B, C, D, ALV-J), CIAV, CELO FADV1, MDV-1, Rev-566 as positive control and clinical samples of DNA as templates. PCR amplification was performed (final 20 µL volume) using 100 ng of each total RNA as template, 1 U Taq DNA polymerase (Invitrogen, [www.lifetech.com](http://www.lifetech.com), Platinum PCR supermix), the selected specific forward and reverse primer ([Supplementary Table 1](#)) (0.25 µM each) and in a buffer containing dNTPs (0.12 mM each), 10 mM Tris–HCl (pH 9.0), 50 mM KCl and 1 mM MgCl<sub>2</sub>. An initial denaturation step was at 94 °C for 5 min, annealing at 55 °C for 2 min and extension at 68 °C for 5 min. The procedure was followed by 35 cycles of 30 s at 94 °C, annealing temperature for 45 s at 50–60 °C (depending on the virus) [Table 1](#), and extension for 1–4 min at 68 °C (depending on the product size). A final cycle consisted of denaturation for 1 min at 94 °C, annealing for 3 min at 55 °C and extension for 10 min at 68 °C. The reactions were cycled in a MJ PCR Peltier thermal Cycle DNA DYAD Engine (M J Inc.). For the RT-PCR procedure RT-PCR amplification was performed (final 20 µL volume) using 100 ng of each total RNA as template, 1 U platinum Taq DNA polymerase (Invitrogen, [www.lifetech.com](http://www.lifetech.com), Superscript III One-step RT-PCR system), the selected specific forward and reverse primer ([Table 1](#)) (0.25 µM each) and in a buffer containing dNTPs (0.4 mM each), 3.2 mM

MgSO<sub>4</sub> and SuperScript RT/Platinum Taq Mix. An initial cDNA synthesis can be achieved in a 60 min incubate at 50 °C. The following denaturation step was at 94 °C for 2 min. The PCR amplification procedure was followed by 40 cycles of 30 s at 94 °C, annealing temperature for 45 s at 50–60 °C (depending on the virus) [Table 1](#), and extension for 1–4 min at 68 °C (depending on the product size). A final cycle consisted of denaturation for 1 min at 94 °C, annealing for 3 min at 55 °C and extension for 10 min at 68 °C. The reactions were cycled in a MJ PCR Peltier thermal Cycle DNA DYAD Engine (M J Inc.). Amplification products from conventional PCR or RT-PCR were separated in 1% TBE (89 mM Tris borate, 2 mM EDTA [pH 8]) agarose gels by using size markers (10 kb, Denville Biochemical) and were visualized under UV illumination by ethidium bromide staining. PCR or RT-PCR products were used for electrophoretic analysis in 1% agarose gels and Ethidium bromide stain was applied to confirm the synthesis of a DNA products or cDNA products of the expected size.

The cell line was tested for Mycoplasma contamination by PCR [34]. Briefly, DNA was extracted from 200 µL of cells and supernatant using the Qiagen DNeasy® Blood and Tissue Kit (QIAGEN, Valencia, CA) according to the manufacturer's instructions. Six PCR protocols were conducted on the purified DNA targeting common avian mycoplasma species as well as mycoplasma in general. The protocols were conducted as previously described for *Mycoplasma gallisepticum* [35], *Mycoplasma synoviae* [36], *Mycoplasma meleagridis* [36], *Mycoplasma iowae* [36], and *Mycoplasma* spp. [37,38].

### 2.3. Viruses

In all assays used in this study for detection of ALV and REV, CEFs from both of ADOL line 0 and RFS inoculated with strain RAV-1 of subgroup A ALV, strain Hc1 of ALV-J, strain RAV-0 of ALV-E, and strain spleen necrosis virus (SNV) of REV viruses were used as positive controls.

Experiments were performed in BSL-2E containment using a lentogenic recombinant Newcastle Disease Virus Lasota strain with red fluorescent protein (LS-RFP) [39] and a wild type Lasota (LS-WT) strain. The virus working stock was produced in 9–11 day-old SPF chicken embryonating eggs kept at the Southeast Poultry Research Laboratory facility using standard methods [1]. Stock viruses were aliquoted in 1 mL volumes with titers of  $3.2 \times 10^9$  EID<sub>50</sub>/mL and  $5.0 \times 10^9$  EID<sub>50</sub>/mL for LS-RFP and LS-WT respectively and stored at –80 °C until use. Each stock was thawed not more than 2 times of use. The viruses were not adapted to the cell line before the experiment in order to avoid altering their antigenicity.

### 2.4. Virus production

#### 2.4.1. Permissiveness of the SciPC to NDV

Initial analysis of the permissiveness of the BA3 cells was carried out using LS-RFP [39]. Cells growing at exponential phase were infected with LS-RFP at multiplicity of infection (MOI) of 5 and adsorbed for 1 h in incubator at 37 °C in 5% atmosphere on a shaker at 50–100 rpm. The cells were washed once and reconstituted with DMEM/F12 supplemented with 5% KSR and incubated for 48 h. Samples were taken at 12 h interval for cell counting. The infected cells were observed under fluorescence microscope.

#### 2.4.2. Newcastle virus vaccine production

Virus infection for vaccine production was performed using NDV (LS-WT). An initial cell seeding density of  $7 \times 10^5$  cells/mL in 5% KSR medium (DMEM/F12 with and without 15 mM HEPES) was used. Infections were done with both 5 and 20% KSR supplementation. Similarly, infection was performed in HyQ SFM4MegaVir (HyClone, Logan, UT), supplemented according to manufacturers' instructions

**Table 1**

Results from screening BA3 cells for freedom of avian leukosis and reticuloendotheliosis viruses. Negative (–).

Sample <sup>a</sup>	Assay	Result <sup>b</sup>			
Supernatant fluids directly from BA3 culture	Direct ELISA for ALV (p27)	–			
Supernatant fluids from ADOL CEFs inoculated with supernatant fluids from BA3 cells	Virus isolation (a)	ELISA ALV gs (p27) antigen			
	Line 0	–			
	RFS	–			
Supernatant fluids from ADOL CEFs after co-cultivation of BA3 cells	Virus isolation (b)	ELISA ALV gs (p27) antigen			
	Line 0	–			
	RFS	–			
	Virus isolation-PCR for ALV/REV	PCR ALV-A	ALV-E	ALV-J	REV
	Line 0	–	–	–	–
	RFS	–	–	–	–

<sup>a</sup> Supernatant fluids from ADOL CEFs (line 0 and RFS) that had been inoculated with supernatant fluids from BA3, or after co-cultivation of BA3 with ADOL CEFs. Supernatant fluids were collected 7–9 day post-inoculation.

<sup>b</sup> As determined by ELISA for the presence of ALV gs (p27) antigen, or by presence of specific PCR products using specific primers. As expected, all control positive samples tested positive by ELISA and PCR (data not shown).

as well as supplementation using 20% KSR for comparison of virus yield and cost effectiveness with DMEM/F12. Infection was carried out at high cell densities at multiple MOIs ranging from 0.1 to 5. In cases where lower MOI (0.1–1) was used, 10 µg/mL of porcine trypsin was added to the medium. All infections were conducted in triplicate and reproduced at minimum of two independent times. Culture plates containing infected cells were incubated at 37 °C on a shaker at 100 rpm in 5% CO<sub>2</sub> incubator for 24–72 h. Viruses were harvested from cells utilizing three freeze–thaw cycles with stocks stored at –80 °C until analyzed.

### 2.5. Virus titration

All virus titrations were performed in 9–11 day-old SPF eggs in order to have the direct comparison and correct titer rather than converting from plaque forming unit (PFU) or tissue culture infective dose (TCID<sub>50</sub>) if cells were to be used. Results of the titration were analyzed using Spearman [40] and Kärber [41] method.

### 2.6. Statistical analysis

Means from multiple groups in the experiment were analyzed by ANOVA with Tukey post hoc test. For all tests, significance was reported at the level of  $P \leq 0.05$ .

## 3. Results

### 3.1. Adaptation of chicken induced pluripotent cells to suspension growth in KSR medium

To improve the ability of chicken induced pluripotent cell cultures to produce higher titers of vaccine virus, we attempted to transition cells from an anchorage dependent culture system to a suspension culture system. Additionally, we attempted to transition these cells to a more cost effective media that do not have the expensive bFGF growth factor initially used to derive these cells or KSR [27]. ciPCs were successfully transitioned by repeated passages from growing as anchorage dependent to suspension culture. In addition cells were simultaneously transitioned to grow from an expensive media containing bFGF to a simple, cost effective serum free media. As shown in Fig. 1A, it took approximately 120 days to generate a suspension ciPC line capable of growth in low KSR medium without bFGF. Once fully adapted to this medium, the cells were no longer adherent and grew as cell clumps of 3–20 cells in suspension. To investigate the optimum concentration of KSR required for the effective growth of ciPC in the medium, gradual

reduction in the concentration KSR was done from 20% to 1%. The concentration of the KSR was decreased by 2% in each step of the adaptation process from 20% to 6%, and cells were allowed to grow in the medium for up to three passages before changing the concentration. The cells were sensitive to changes in low KSR concentration hence, higher cell death was seen when KSR was changed from 6% to 4%. Based on this observation, subsequent adaptation was carried out with 1% reduction in KSR concentration for each step until the KSR level was as low as 1%. At KSR concentration of lower than 5%, the medium does not effectively support the growth of ciPCs. The cell line was passed in 5% KSR medium for more than 100 generations. The cell thrives best at 20, 10 and 5% KSR (Fig. 1C–E). Though, the cells are capable of growth at lower KSR concentrations but slowly and sparsely (Fig. 1F and G).

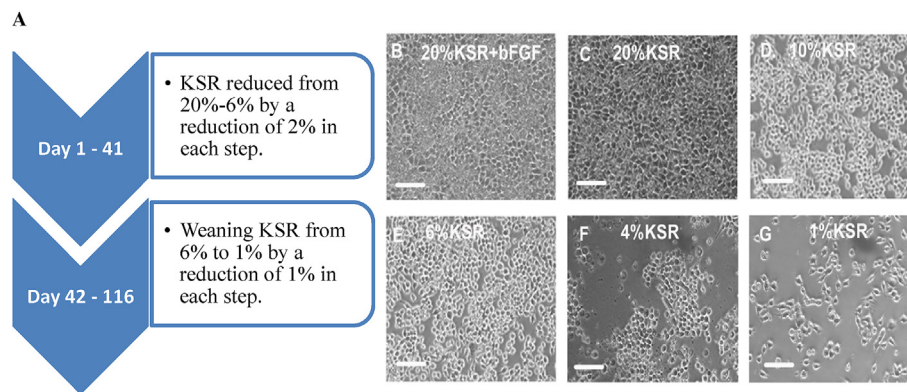
### 3.2. Cell growth kinetics

To determine the maximum number of cells that could be grown per mL in suspension culture, a growth study was performed using DMEM/F12 with and without HEPES supplemented with 1 and 5% KSR with an initial cell seeding density of  $7.0 \times 10^5$  cells/mL. Comparisons were made between cells grown in DMEM/F12 with and without HEPES. A slower and lower saturation density of  $8.5 \times 10^5$  cells/mL was recorded for the medium supplemented with 1% KSR (data not shown) (Fig. 2). On the contrary, cells grown with medium supplemented with 5% KSR grew faster and to a higher saturation density of  $6.0\text{--}7.0 \times 10^6$  cells/mL after 144 h with daily medium change (Fig. 3). No significant difference was noticed when cells were grown in DMEM/F12 medium with ( $6.0\text{--}7.0 \times 10^6$  cells/mL) or without HEPES ( $6.0 \times 10^6$  cells/mL). This shows that DMEM/F12 with HEPES may not increase the cell number significantly and the changes in pH are not likely sufficient to affect growth under the condition tested. Doubling time of approximately 39 and 21 h were recorded for cells grown with 1% and 5% KSR respectively. Based on these results, the 5% KSR medium will be better suited for the production of the cells at industrial level due to faster growth kinetics. The 5% KSR medium supplementation was then used in growth related experiments throughout the study.

### 3.3. Testing for the presence of avian leukosis, reticuloendotheliosis viruses, Fowl adenovirus, Marek's disease virus and mycoplasma

One major potential limiting factor in the use of SciPC cells as substrate for production of avian vaccines is the potential contamination of cell with chicken endogenous and exogenous ALV





**Fig. 1.** Adaptation of BA3 cell line to growth in knock-out serum-replacement medium. (A) Timeline showing the adaptation of BA3 cell line in KSR. (B, C) Proliferation of the BA3 cell line in 20% KSR medium with and without bFGF. (D) BA3 cell line cultured in 10% KSR and (E) in 6% KSR. (F) Survival during reduction from 5% KSR to 4% KSR and (G) in 1% KSR despite reduction in proliferation (scale bar = 50 μm).

and REV. We therefore determined if our cells were contaminated with these viruses. Table 1 shows results obtained from testing ciPC cells for the presence of ALV and REV. Two virus isolation tests (a and b) were conducted on BA3 cell line. CEFs from both ADOL line 0 and RFS tested negative for both endogenous and exogenous ALV, as determined by testing either supernatant obtained directly from BA3 cell line (a), or supernatant fluids from co-cultivation of BA3 cells with CEFs from ADOL line 0 and RFS (b). PCR results from testing DNA extracted from CEFs from ADOL line 0 and RFS inoculated with supernatant fluids from co-cultivation of BA3 cells with ADOL line 0 and RFS are also shown in Table 1. Using primers that detect ALV-A, ALV-E and ALV-J [33] and REV [32], the results show that BA3 cells were negative for the presence of ALV-A, ALV-J, ALV-E, and REV. We also assessed potential contamination of the BA3 line for Fowl adenovirus 1, REV, Marek's disease virus, Mycoplasma and Chicken Infectious Anemia virus by direct PCR or RT-PCR and found the cells to be negative (data not shown). The mycoplasma test included 4 species of avian mycoplasma and one generic test against mammalian species.

#### 3.4. Permissiveness of SciPC cells to infection with NDV LaSota-RFP

It is critical to test the susceptibility of (SciPC) cells to infection by viruses in order to determine their potential for generating vaccine virus. SciPC cells were found to be highly permissive to infection using the NDV Lasota RFP (red fluorescent protein; an engineered marker for the detection of infection) vaccine virus strain. Upon infection with the virus, the cell number ( $4.5 \times 10^6$  cells/mL) was constant for the first 12 h with infected cells showing expression of red fluorescence (Fig. 4B, D and E). An approximately 1.5 fold decrease in cell number ( $3.3 \times 10^6$  cells/mL) was recorded after 24 h post-infection, which correlated with increased fluorescence (Fig. 3B). In contrast, cell numbers of the un-infected control rose with an equivalent fold increase to a maximum of  $7.0 \times 10^6$  cells/mL within the same 24 h time period (Fig. 3B).

#### 3.5. Virus production

To determine the capacity of the suspension cells to produce high levels of infectious vaccine viruses we infected exponentially growing cells in suspension. SciPCs growing at exponential phase in a 6 well plate were infected with LS-WT in the presence of complete medium (DMEM/F12 supplemented with 5 or 20% KSR) changed prior to infection. Before infection, cells grew as clumps of

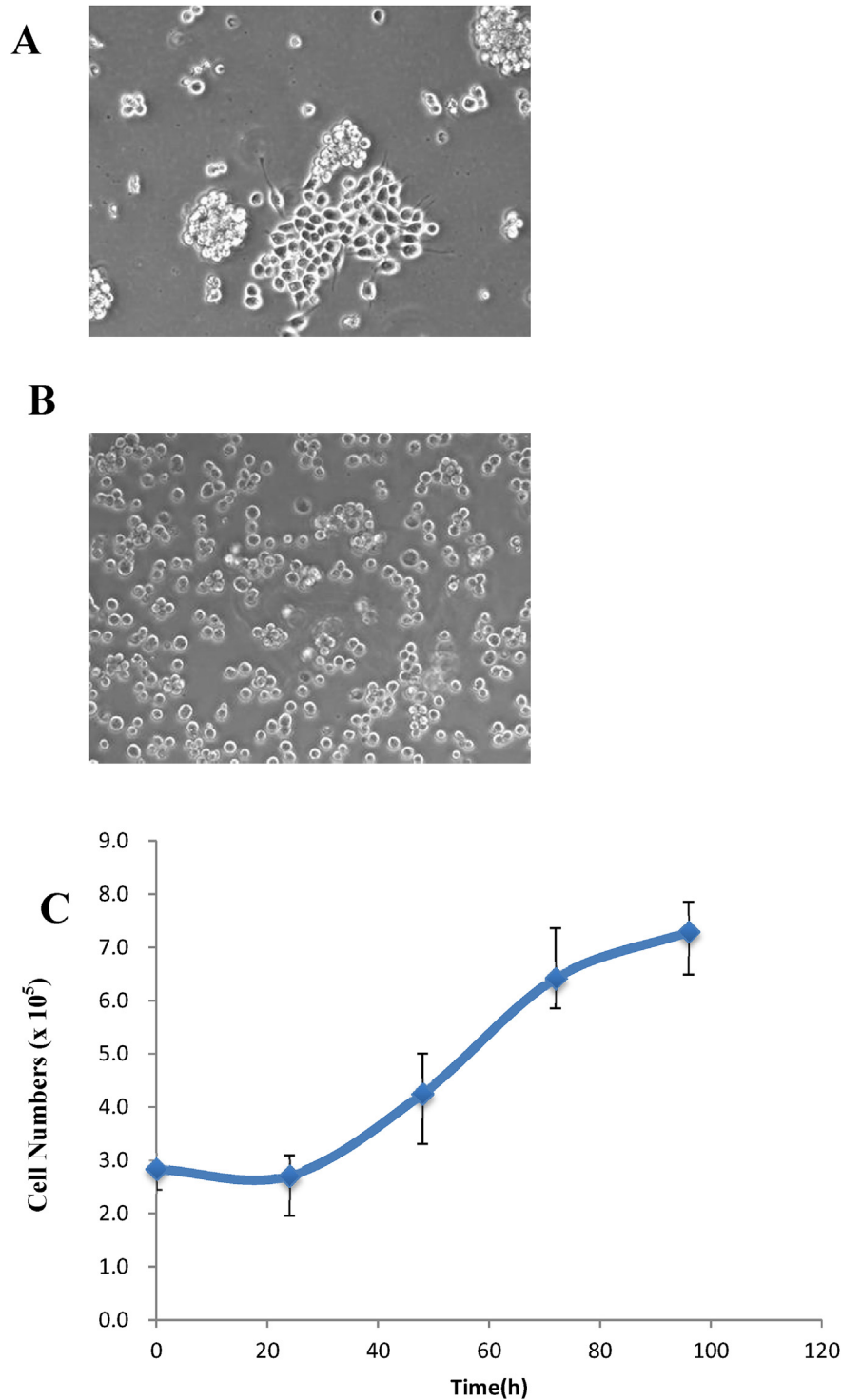
3–20 cells but upon infection the clumps were easily dispersed and the cells remained as single cells. Changes were observed in the morphology of the cells upon infection. By 12 h post infection (PI) fusion of the cells was observed forming giant multi-nucleated cells with decreases in viable cell numbers (Fig. 4E).

To assess if increased cell densities would lead to increased virus titers, three different cell densities ( $3.5 \times 10^6$ ,  $4.5 \times 10^6$  and  $7.0 \times 10^6$  cells/mL) infected at MOI 5 using DMEM/F12 supplemented with 20% KSR were tested (Fig. 5). No significant statistical differences were observed in virus yield for all three different densities at 24 and 48 h post infection. Maximum virus titers of  $9.6 \times 10^7$ ,  $8.3 \times 10^7$  and  $8.6 \times 10^7$  EID<sub>50</sub>/mL were obtained at 24 h post infection for cell densities  $3.5 \times 10^6$ ,  $4.5 \times 10^6$  and  $7.0 \times 10^6$  cells/mL respectively (Fig. 5A). However, statistical differences were found between cell densities ( $4.5 \times 10^6$  and  $7.0 \times 10^6$  cells/mL) infected at MOI 5 using DMEM/F12 supplemented with 5% KSR at 24 and 48 h time points post infection (Fig. 5B). In another experiment, a virus titer of  $1.02 \times 10^8$  EID<sub>50</sub>/mL was recorded at 24 h PI when DMEM/F12 containing 15 mM HEPES supplemented with 20% KSR was used to grow the cell.

To determine if a lower cost media would affect virus yield, a serum free medium used for the production of high virus yield (SFM4MEGAVIR, from Hyclone) was used to study the effect of a different medium on the virus production. Infection of SciPCs growing at exponential phase with MOI 5 using DMEM/F12 containing 15 mM HEPES and SFM4MEGAVIR with and without 20% KSR supplementation was carried out. The highest virus titer of  $1.02 \times 10^8$  was recorded for DMEM/F12 containing 15 mM HEPES supplemented with 20% KSR (Fig. 6). Virus titers of  $3.7 \times 10^7$  EID<sub>50</sub>/mL and  $3.1 \times 10^7$  EID<sub>50</sub>/mL were recorded for SFM4MEGAVIR supplemented with 20% KSR and SFM4MEGAVIR respectively. There is significant difference ( $P < 0.05$ ) in the virus titer obtained using DMEM/F12 against SFM4MEGAVIR with and without 20% KSR supplementation (Fig. 6). These results demonstrate that change of medium with SFM4MEGAVIR did not positively impact on virus yield in comparison with DMEM/F12 medium, however virus yield we still relatively high.

## 4. Discussion

In this study, we demonstrated that BA3 is an immortal cell line derived from CEF by a mini-circle DNA reprogramming approach [27]. We also demonstrated that this ciPCs could be transitioned to suspension cultures system utilizing on a cost saving media without bFGF and reduced levels of KSR. In addition, the SciPC cell

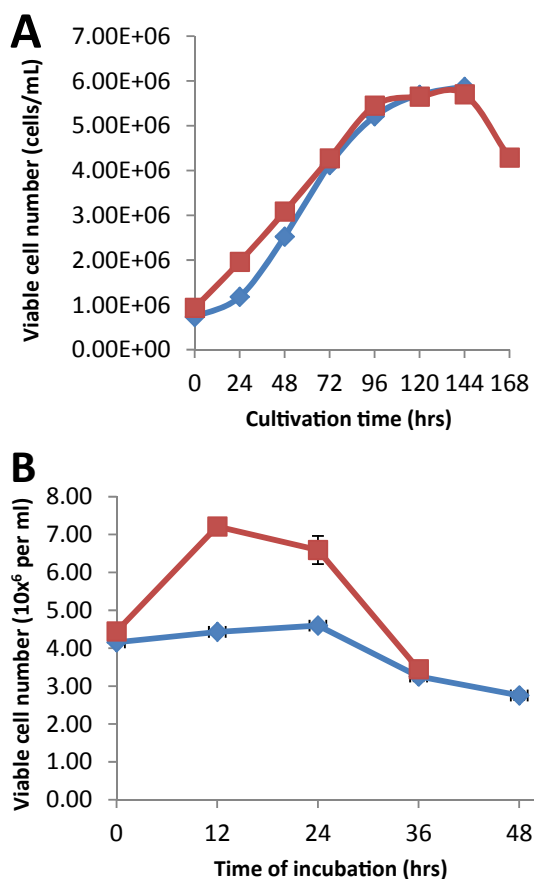


**Fig. 2.** BA3 cell line proliferation in suspension culture. (A and B) BA3 cell line grown in petri-dish using 1% KSR before and after manual breaking of the cell clumps, respectively. (C) Doubling time of the cells in suspension is 39 h and grows to density of  $8.5 \times 10^5/\text{mL}$  (scale bar A, B: 100  $\mu\text{m}$ ).

line BA3 was found to be free of adventitious agents and its growth dynamics were found to be suitable for production of a ND virus vaccine. Virus isolation assays using CEFs for detection of ALV and REV in various samples are considered to be the golden standard [42]. Using this method suspected viruses are allowed to propagate and amplify for 7–10 days in susceptible CEFs before testing the inoculated cells for the presence of ALV gs antigen (p27) by ELISA, or by testing DNA extracted from such cells by PCR using primers

for various subgroups of ALV. Similarly, testing DNA extracted from inoculated CEFs for the presence of REV using specific primers has been shown to be specific and sensitive method for detection of REV [32].

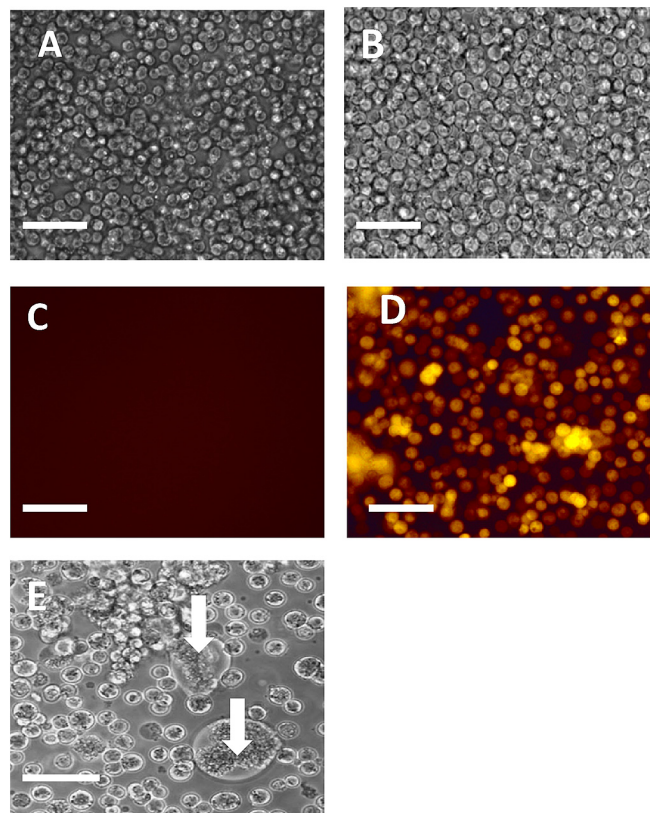
The finding that CEFs from ADOL lines 0 (C/E, cells that are resistant to ALV-E) and RFS (C/O, cells that are susceptible to all subgroups of ALV) inoculated with supernatant fluids obtained directly from BA3 cells, or after co-cultivation of BA3 cells with CEFs



**Fig. 3.** Growth curves of BA3 cells before and after infection with Lasota-RFP. (A) BA3 cells were grown in DMEM/F12 with (red line) and without (blue line) 15 mM HEPES buffer supplemented with 5% KSR data collection at 24 h interval. (B) Progression of cell growth during infection with Lasota-RFP at MOI of 5 with starting cell density of  $4.5 \times 10^6$  EID<sub>50</sub>/mL (blue), and un-infected control cells (red). Data represent mean values for two independent experiments carried out in triplicate.

from line 0 and RFS tested negative for ALV gs antigen (p27) indicate that BA3 cells are free from complete infectious exogenous and endogenous ALV. Also, the finding that DNA extracted from CEFs inoculated with BA3 tested negative for the presence of ALV-A, ALV-E and ALV-J by PCR confirmed the virus isolation ELISA results. DNA extracted from CEFs inoculated with supernatant fluids from BA3 also tested negative for the presence of REV by PCR, indicating that BA3 preparations tested in this study are free from REV. It is important to note that all substrates, chicken embryos, or CEFs used for production of live-virus vaccines are required to be free from avian retroviruses, namely ALV and REV [43]. Our results clearly show that BA3 cells are free from such ALV and REV and could be used for production of live virus vaccine of poultry.

The transition of BA3 from adherent to suspension culture was successfully accomplished alongside the optimum concentration of KSR. SciPCs thrive optimally when grown in DMEM/F12 with or without HEPES buffer using 5% KSR supplementation making it a serum free medium. This adaptation should help cutting down on the cost of production with flexibility for usage from using 20% KSR supplementation to 5% without significantly affecting the results. The use of KSR instead of FBS has the additional benefit that reduces the growth as clumps of 5–20 cells and it takes little effort to break up the clumps when passing of cells is required, cutting down on the cost for procuring digestion enzymes. In general,

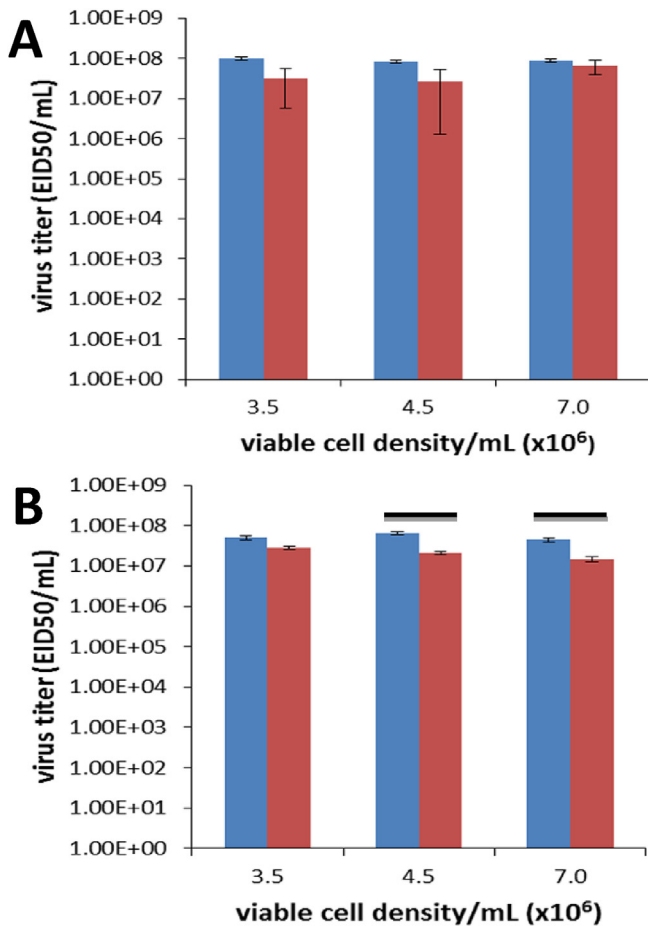


**Fig. 4.** Morphology of BA3 cells. BA3 cells were infected with Lasota-RFP at MOI of 5 and adsorbed for 1 h at 37 °C in 5% CO<sub>2</sub> incubator. The cells were viewed starting from 12 h post-infection. (A) Bright field of un-infected cells observed at 20 $\times$  magnification. (B) Bright field of infected cells with Lasota-RFP at MOI 5. (C) Fluorescence field view of un-infected cells. (D) Fluorescence field view of infected cells at MOI 5 with Lasota-RFP. (E) Infection of cells with MOI 5 at 24 h post-infection with LS-WT. White arrows show fused cell forming giant multi-nucleated cells (scale bar = 100  $\mu$ m). Data represent two independent experiments generated after 12 h of cell seeding and infection (scale bar = 100  $\mu$ m).

demonstration of the adaptability and scalability of other types of iPC has been previously described [44,45]. Here upon seeding, the chicken origin cell line BA3 cell showed a short lag phase and entered the logarithmic phase within 24–48 h (Fig. 3A). The short doubling time makes BA3 a prime candidate for easy scale up with the cells being ready for infection immediately after seeding. Comparable values were observed when cells were grown in DMEM/F12 with or without HEPES supplemented with 5% KSR. The SciPCs attained maximum cell concentration of  $6.0\text{--}7.0 \times 10^6$  cells/mL in a 6 well plate within 144 h post seeding.

In addition to industrial friendly features, the chicken origin of SciPCs provides significant advantages over existing systems. Similar virus yields were observed for embryonic stem cells generated from duck cell line reported previously [24,46]. Attempts by several investigators at adapting MDCK and Vero to growing in serum free medium as suspension culture have yielded cell concentrations of  $1.2 \times 10^6$  cells/mL [19],  $2.2 \times 10^6$  cells/mL [22] and  $2.5 \times 10^6$  cells/mL [21]. These titers values are lower in comparison to our result, but most important the used cells of mammalian origin carry the risk of mutation, attenuation and adaptation of avian viruses to mammals.

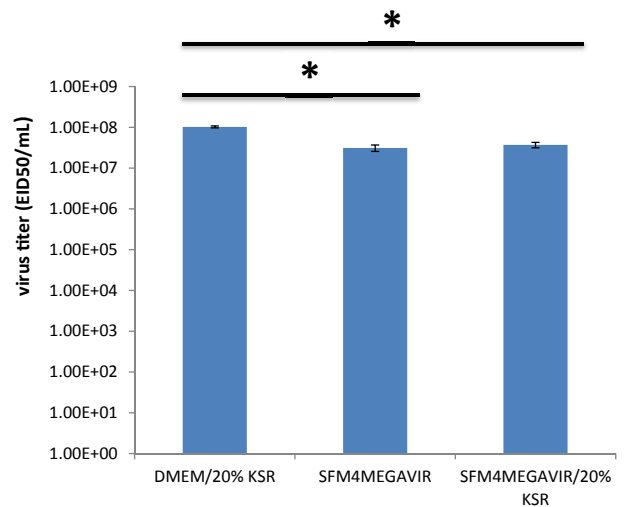
Cells designed for industrial vaccine production should be easily scalable and grow fast to meet the production demand as required. The optimization step for the Newcastle disease virus production involved looking at the infection of BA3 cell at different density,



**Fig. 5.** Virus titers produced by three different cell densities of BA3 cell line grown in DMEM supplemented with 20 and 5% KSR and infected with Newcastle disease virus LaSota vaccine at MOI of 5. Infected cells were adsorbed for 1 h at 37 °C in 5% CO<sub>2</sub> incubator with medium changed post adsorption to DMEM supplemented with 20% and 5% KSR (A) virus titers for 20% KSR at 24 (blue bars) and 48 h (red bars) post infection. No significant statistical differences were found between different cell densities and time points (24 and 48 h). (B) Virus titers for 5% KSR at 24 and 48 h PI. Horizontal bar with asterisk shows significant statistical differences between 24 and 48 h at densities of 4.5 and 7.0 × 10<sup>6</sup>/mL.

concentration of KSR, medium changes, and time post infection. The first part of the experiments involved the use of Newcastle disease LS-RFP to assess the permissiveness of BA3. It was observed that Newcastle disease virus at MOI 5 infected and replicated in over 90% of the infected cells suggesting that the cell is highly permissive for the virus. When NDV Lasota was used at the same MOI maximum virus titer of  $1.02 \times 10^8$  EID<sub>50</sub>/mL was obtained at 24 h PI. It has been observed that infection at high MOI results in synchronous virus growth and early peak of virus titer [47].

Influence of cell density on Newcastle disease virus yield was assessed by infecting cells at 3 different densities (3.5, 4.5 and 7.0 × 10<sup>6</sup> cells/mL). Cell density is an important factor in vaccine production. Cells growing to higher concentration offer an advantage in terms of availability of cells per volume for virus infection, hence higher titer. The limitation of this as reported by Bernal et al. [48] has been that at certain cell concentration threshold there is a decrease in the number of virus particles being produced, referred to as the “cell density effect”. The cause of this effect has been attributed to be the depletion of nutrients or accumulation of inhibiting substances [49]. The virus titers (Fig. 5A) obtained at 24 h PI for BA3 cells infected at various cell densities were comparable



**Fig. 6.** Virus titers produced by BA3 cell lines grown in DMEM/F12 and SFM4MEGAVIR media after infection with Newcastle Disease LaSota. BA3 SciPCs grown in 5% KSR medium were infected at MOI 5 and adsorbed for 1 h at 37 °C in 5% CO<sub>2</sub> incubator. Medium was changed post adsorption to DMEM/F12 supplemented with 20% KSR, SFM4MEGAVIR and SFM4MEGAVIR supplemented with 20% KSR and incubated on shaker at 37 °C in 5% CO<sub>2</sub> incubator. Supernatant from infected cell culture was collected 24 h post-infection and subjected to three freeze–thaw cycle before virus titer was determined by EID<sub>50</sub> assay. The virus titer is an average of two independent experiments with samples run in triplicate per experiment. Horizontal bar with asterisk shows significant statistical differences in virus yield between DMEM/F12 supplemented with 20% KSR and SFM4MEGAVIR supplemented with or without 20% KSR.

among the different groups indicating “no cell density effect”. The result of this study is consistent with the observations reported by Lohr et al. [46] who also observed no “cell density effect” for the AGE1.CR.pIX cell line.

A maximum achievable titer of  $3.7 \times 10^7$  EID<sub>50</sub>/mL was recorded when SFM4MEGAVIR supplemented with 20% KSR Medium was used. The value was lower than that obtained when DMEM/F12 was used under the same condition. A similar trend was observed when SFM4MEGAVIR was used for the growth of Vero cell in Influenza virus production [19]. The reason for this could be that the BA3 has been adapted to growing in DMEM/F12 and require adaptation in SFM4MEGAVIR for effective growth. The constituent of the two media could be another possible reason for the low virus yield.

## 5. Conclusion

In this study, we developed and adapted a SciPC cell line to growth in suspension culture. The growth characteristic of SciPCs and freedom from adventitious agents were established. To assess the susceptibility of the cell to virus infection, Newcastle disease virus Lasota strain was used and the virus replicated in the cell to high titer in a relatively short time. With this cell line it is possible that several cycles of the virus production may be achieved, however further adaptation and testing is likely to be needed before these cells are utilized as a viable replacement of embryonating eggs. This data has shown the potential of the SciPC cell line as a candidate for Newcastle disease virus production.

## Acknowledgments

The authors would like to Dr. Jody Mays and Dawn Williams-Coplin for their excellent technical assistance. This work was



supported by GALVmed, Edinburgh, and by USDA CRIS projects 6612-32000-064-00D and 5050-32000-016-00D.

## Appendix A. Supplementary data

Supplementary data related to this article can be found at <http://dx.doi.org/10.1016/j.biologicals.2015.09.002>.

## References

- [1] OIE. Newcastle disease. In: World Organization for Animal Health, editor. *Man. diagnostic tests vaccines terr. anim.*. 4th ed. Paris: France; 2004. p. 270–82.
- [2] Awan MA, Otte MJ, James A. The epidemiology of Newcastle disease in rural poultry: a review. *Avian Pathol* 1994;23:405–23.
- [3] Alexander DJ. Newcastle disease and avian paramyxovirus infections. In: Calnek BW, Barnes HJ, Beard CW, McDougald LR, Saif YM, editors. *Diseases of poultry*. 10th ed. Ames, IA: Iowa State University Press; 1997. p. 541–69.
- [4] Chimeno Zoth S, Gómez E, Carrillo E, Berinstein A. Locally produced mucosal IgG in chickens immunized with conventional vaccines for Newcastle disease virus. *Braz J Med Biol Res* 2008;41:318–23.
- [5] Ewert DLL, Barger BO, Eidson CS. Local antibody response in chickens: analysis of antibody synthesis to Newcastle disease virus by solid-phase radioimmunoassay and immunofluorescence with class-specific antibody for chicken immunoglobulins. *Infect Immun* 1979;24:269–75.
- [6] Zakay-Rones Z, Levy R. Immunologic response of chicks to inactivated Newcastle disease virus. *Avian Dis* 1973;17:450–2.
- [7] Cho S, Kwon H, Kim T-E, Kim J-H, Yoo H, Park M-H, et al. Characterization of a recombinant Newcastle disease virus vaccine strain. *Clin Vaccine Immunol* 2008;15:1572–9.
- [8] Huang Z, Elankumaran S, Panda A, Samal SK. Recombinant Newcastle disease virus as a vaccine vector. *Poult Sci* 2003;82:899–906.
- [9] Kim S-HH, Wanasen N, Paldurai A, Xiao S, Collins PL, Samal SK. Newcastle disease virus fusion protein is the major contributor to protective immunity of genotype-matched vaccine. *PLoS One* 2013;8:e74022.
- [10] Cardenas-Garcia S, Diel DG, Susta L, Lucio-Decanini E, Yu Q, Brown CC, et al. Development of an improved vaccine evaluation protocol to compare the efficacy of Newcastle disease vaccines. *Biologicals* 2015;43:136–45.
- [11] Rattanani J. India to see tremendous changes. *World Poult* 2006;10–2. [www.worldpoultry.net/PageFiles/.../001\\_boerderij-download-WP6524D01.pdf](http://www.worldpoultry.net/PageFiles/.../001_boerderij-download-WP6524D01.pdf) [accessed 02.03.15].
- [12] Liu J, Shi X, Schwartz R, Kemble G. Use of MDCK cells for production of live attenuated influenza vaccine. *Vaccine* 2009;27:6460–3.
- [13] Hussain AI, Cordeiro M, Sevilla E, Liu J. Comparison of egg and high yielding MDCK cell-derived live attenuated influenza virus for commercial production of trivalent influenza vaccine: in vitro cell susceptibility and influenza virus replication kinetics in permissive and semi-permissive cells. *Vaccine* 2010;28:3848–55.
- [14] Ehrlich HJ, Singer J, Berezuk G, Fritsch S, Aichinger G, Hart MK, et al. A cell culture-derived influenza vaccine provides consistent protection against infection and reduces the duration and severity of disease in infected individuals. *Clin Infect Dis* 2012;54:946–54.
- [15] Arifin MA, Mel M, Abdul Karim MI, Ideris A. Production of Newcastle disease virus by vero cells grown on cytodex 1 microcarriers in a 2-litre stirred tank bioreactor. *J Biomed Biotechnol* 2010;2010:1–7.
- [16] Mohd Azmir Arifin. Optimization of Newcastle disease virus production in T-flask. *Afr J Biotechnol* 2011;10:18816–23.
- [17] Terregino C, Capua I. Clinical traits and pathology of Newcastle disease infection and guidelines for farm visit and differential diagnosis. In: Capua I, Alexander DJ, editors. *Avian influenza and Newcastle disease. A field laboratory manual*. Milano: Springer Milan; 2009. p. 113–22.
- [18] Moran E. A microcarrier-based cell culture process for the production of a bovine respiratory syncytial virus vaccine. *Cytotechnology* 1999;29:135–48.
- [19] Chen A, Poh SL, Dietzsch C, Roethl E, Yan ML, Ng SK. Serum-free microcarrier based production of replication deficient influenza vaccine candidate virus lacking NS1 using Vero cells. *BMC Biotechnol* 2011;11:81.
- [20] Genzel Y, Olmer RMM, Schäfer B, Reichl U. Wave microcarrier cultivation of MDCK cells for influenza virus production in serum containing and serum-free media. *Vaccine* 2006;24:6074–87.
- [21] Paillet C, Forno G, Kratje R, Etcheverrigaray M. Suspension-Vero cell cultures as a platform for viral vaccine production. *Vaccine* 2009;27:6464–7.
- [22] Van Wielink R, Kant-Eenbergen HCMCM, Harmsen MMM, Martens DEE, Wijffels RHH, Coco-Martin JMM. Adaptation of a Madin–Darby canine kidney cell line to suspension growth in serum-free media and comparison of its ability to produce avian influenza virus to Vero and BHK21 cell lines. *J Virol Methods* 2011;171:53–60.
- [23] Pau MGG, Ophorst C, Koldijk MHH, Schouten G, Mehtali M, Uytendaele F. The human cell line PER.C6 provides a new manufacturing system for the production of influenza vaccines. *Vaccine* 2001;19:2716–21.
- [24] Mehtali M, Chamion-Arnaud P, Leon A. Process of manufacturing viral vaccines in suspension avian embryonic derived stem cell lines. WO 2006/108846 A1; 2006.
- [25] Jordan I, Vos A, Beilfuss S, Neubert A, Breul S, Sandig V. An avian cell line designed for production of highly attenuated viruses. *Vaccine* 2009;27:748–56.
- [26] Genzel Y, Behrendt I, Rödiger J, Rapp E, Kueppers C, Kochanek S, et al. CAP, a new human suspension cell line for influenza virus production. *Appl Microbiol Biotechnol* 2013;97:111–22.
- [27] Yu P, Lu Y, Jordan BJ, Liu Y, Yang J-Y, Hutcheson JM, et al. Nonviral minicircle generation of induced pluripotent stem cells compatible with production of chimeric chickens. *Cell Reprogram* 2014;16:366–78.
- [28] Jia F, Wilson KD, Sun N, Gupta DM, Huang M, Li Z, et al. A nonviral minicircle vector for deriving human iPS cells. *Nat Methods* 2010;7:197–9.
- [29] Lu Y, West F, Jordan B, Jordan E, West R, Yu P, et al. Induced pluripotency in chicken embryonic fibroblast results in a germ cell fate. *Stem Cells Dev* 2014;23:1755–64.
- [30] Crittenden LB, McMahon S, Halpern MS, Fadly AM. Embryonic infection with the endogenous avian leukosis virus Rous-associated virus-0 alters response to exogenous avian leukosis virus infection. *J Virol* 1987;61:722–5.
- [31] Zhang H, Bacon LD, Fadly AM. Development of an endogenous virus-free line of chickens susceptible to all subgroups of avian leukosis virus. *Avian Dis* 2008;52:412–8.
- [32] Aly MMM, Smith EJJ, Fadly AM. Detection of reticuloendotheliosis virus infection using the polymerase chain reaction. *Avian Pathol* 1993;22:543–54.
- [33] Silva RF, Fadly AM, Taylor SP. Development of a polymerase chain reaction to differentiate avian leukosis virus (ALV) subgroups: detection of an ALV contaminant in commercial Marek's disease vaccines. *Avian Dis* 2007;51:663–7.
- [34] Kleven S. Mycoplasmosis. In: Dufour-Zavala L, Swayne DE, Glisson JR, Pearson JE, Reed WM, Jackwood MW, et al., editors. *A laboratory manual for the isolation, identification and characterization of avian pathogens*. 5th ed. Athens, GA: American Association of Avian Pathologists; 2008. p. 59–64.
- [35] Callison SA, Riblet SM, Sun S, Ikuta N, Hilt D, Leiting V, et al. Development and validation of a real-time Taqman polymerase chain reaction assay for the detection of *Mycoplasma gallisepticum* in naturally infected birds. *Avian Dis* 2006;50:537–44.
- [36] Raviv Z, Kleven SH. The development of diagnostic real-time TaqMan PCRs for the four pathogenic avian mycoplasmas. *Avian Dis* 2009;53:103–7.
- [37] Ramírez AS, Naylor CJ, Pitcher DG, Bradbury JM. High inter-species and low intra-species variation in 16S–23S rDNA spacer sequences of pathogenic avian mycoplasmas offers potential use as a diagnostic tool. *Vet Microbiol* 2008;128:279–87.
- [38] Johansson KE. PCR based on 16S rRNA sequences for detection of mycoplasmas. *Pharmeuropa* 2000:45–56.
- [39] Li J, Hu H, Yu Q, Diel DG, Li D, Miller PJ. Generation and characterization of a recombinant Newcastle disease virus expressing the red fluorescent protein for use in co-infection studies. *Virol J* 2012;9:227.
- [40] Spearman C. The method of “right and wrong cases” (“constant stimuli”) without Gauss's formulae. *Br J Psychol* 1908;227–42.
- [41] Kärber G. Beitrag zur kollektiven Behandlung pharmakologischer Reihenversuche. *Arch Exp Pathol Pharmacol* 1931;162:480–3.
- [42] Fadly AM, Witter RL, Hunt HD. Oncornaviruses: leukosis/sarcoma and reticuloendotheliosis. In: Glisson JR, Jackwood MW, Pearson JE, Reed WM, Swayne DE, editors. *A laboratory manual for the isolation, identification and characterization of avian pathogens*. 5th ed. American Association of Avian Pathologists; 2008. p. 101–10.
- [43] WHO Expert Committee on Biological Standardization. Requirements for measles, mumps and rubella vaccines and combined vaccines (live). Requirements for biological substances, no. 47. *World Health Organ Tech Rep Ser* 1994;840:100–207.
- [44] Zweigert R, Olmer R, Singh H, Haverich A, Martin U. Scalable expansion of human pluripotent stem cells in suspension culture. *Nat Protoc* 2011;6:689–700.
- [45] Wang Y, Chou B-K, Dowey S, He C, Gerecht S, Cheng L. Scalable expansion of human induced pluripotent stem cells in the defined xeno-free E8 medium under adherent and suspension culture conditions. *Stem Cell Res* 2013;11:1103–16.
- [46] Lohr V, Genzel Y, Jordan I, Katering D, Mahr S, Sandig V, et al. Live attenuated influenza viruses produced in a suspension process with avian AGE1.CR.pIX cells. *BMC Biotechnol* 2012;12:79.
- [47] Audsley JM, Tannock GA. The growth of attenuated influenza vaccine donor strains in continuous cell lines. *J Virol Methods* 2005;123:187–93.
- [48] Bernal N, Carinhas N, Yokomizo AY, Carrondo MJT, Alves PM. Cell density effect in the baculovirus-insect cells system: a quantitative analysis of energetic metabolism. *Biotechnol Bioeng* 2009;104:162–80.
- [49] Meghrouh J, Mahmoud W, Jacob D, Chubet R, Cox M, Kamen AA. Development of a simple and high-yielding fed-batch process for the production of influenza vaccines. *Vaccine* 2009;28:309–16.