Propagation Properties of a New Human Diploid Cell Line, RAZI-HDC, and Its Suitability as a Candidate Cell Substrate for Respiratory Syncytial Virus Vaccine Production in Comparison to MRC-5

Abstract

Respiratory syncytial virus is a common cause of infection of the respiratory tract in infants, older adults, individuals with heart and lung disease, and immunocompromised patients. The disease causes between 100,000 and 200,000 infant deaths annually.

Several vaccine platforms have been introduced for RSV vaccine production. In this study, a local diploid cell line, RAZI-HDC, derived from human fetal lung cells, was used for RSV virus propagation regarding to study live-attenuated vaccine, and was compared to the MRC-5 cell line.

The total cells per 25cm2 flask were 44.0 ± 2.6 *10^5 and 41.66±2.08 *10^5 for MRC-5 and RAZI-HDC, respectively. The maximum cell-specific growth rate of RAZI-HDC was 316.66±20.81, while that of MRC-5 was only 340±26.45. The maximum cell division number of RAZI-HDC was 1.24±0.07 in comparison to the MRC-5, with a maximum cell division number of 1.32±0.08. Both cell substrates achieved maximum cell density 5 days after starting the culture. The complete cytopathic effect of RSV in RAZI-HDCR-HDC was observed after four days, which indicates the sensitivity of these cells to RSV. The virus productivity in RAZI-HDC cells (2.4685) was not significantly different from that in MRC-5 cells (2.5), as determined by a two-tailed t-test (p=0.78). The results showed that both cell substrates have the same function for RSV propagation. Diploid cell lines like MRC-5 and RAZI-HDC are preferred for vaccine manufacturing as they are of human origin and have a stable karyotype. This is a significant advantage, as it helps ensure the safety of the final vaccine product if these cells are used to make viral vaccines that require virus

amplification. The ability of RAZI-HDC cell line in supporting the RSV replication, were assessed and found to be equivalent to those of MRC-5. Specifically, the maximum virus productivity in RAZI-HDC cells (2.4685 log TCID50/mL) was not significantly different from that in MRC-5 cells (2.5 log TCID50/mL), as determined by statistical analysis. Using a locally developed cell line like RAZI-HDC can be somewhat more cost-effective than relying on imported cell substrates.

Keywords: RSV, propagation, RAZI-HDC, MRC-5 cell line, vaccine.

1. Introduction

Respiratory Syncytial Virus (RSV) is the primary cause of acute respiratory infection (ARI) in both infants and older adults, leading to the majority of hospitalisations and the second-highest number of deaths in children under five due to lower respiratory tract infections worldwide (1).

RSV infection is virtually universal among children by the age of two, with frequent reinfections. In adults, RSV is a significant cause of community-acquired pneumonia, particularly during the winter months, with the potential for progression to respiratory failure or death (2). The substantial infection rate and risk of severe complications underscore RSV as a significant public health challenge and a substantial economic burden. Consequently, a safe and effective vaccine could significantly mitigate severe outcomes, decrease hospitalizations and fatalities, and enhance the health and well-being of children and at-risk populations.

RSV belongs to the pneumovirus genus within the Paramyxoviridae family. It is an enveloped virus possessing a negative-sense, single-stranded RNA genome, ranging from 15,191 to 15,226 nucleotides, which encodes for 11 proteins (3). The virus's main immunogens, the surface glycoproteins F and G, elicit neutralising antibodies that inhibit viral fusion and binding, respectively. RSV is divided into two antigenic groups, A and B, distinguished by variations in

glycoprotein G and other proteins (4). Most licensed human viral vaccines, such as those for measles, mumps, rubella, rotavirus, varicella, yellow fever, and influenza (nasal), are live attenuated virus forms (5). .

Live attenuated vaccines (LAVs) represent a promising approach to immunizing children and adults against RSV without inducing the disease. These vaccines mimic natural infections and are expected to confer active immunization by stimulating innate and adaptive immune responses. Such vaccines exhibit limited replication, are well-tolerated, and are the only RSV vaccines demonstrated to be safe in RSV-naive subjects through studies (6). The development of LAVs involved selecting viral mutants adapted at low temperatures (cold passage) or through mutagenesis and selection for viruses incapable of thriving at higher temperatures (temperature-sensitive mutants) (7). This strategy allows the mutants to replicate in the upper respiratory tract while preventing growth in the lower respiratory tract, thus avoiding disease (8).

A key goal in developing attenuated RSV vaccines is to identify viral mutants that maintain a delicate balance: sufficiently attenuated to avoid causing disease yet robust enough to stimulate the immune system and confer immunity effectively. The advancement of LAVs has been enabled by reverse genetics, which allows precise modifications and deletions in the RSV genome (9). However, it is crucial to consider that the virus's passage can influence its evolution, and the reverse genetics approach entails artificial manipulation of the genetic material to create mutations outside of natural evolutionary processes (10).

An appropriate cell line for virus passage is critical in RSV vaccine development. The chosen cell line must support efficient virus replication and be safe for humans. Historically, vaccines have been developed using various cell substrates, including primary cultures from adult animals, bird tissues, and cell lines. Although primary cultures from adult animals are being phased

out due to severe side effects, bird tissue substrates present fewer side effects but may still trigger local allergic reactions and lower antibody titers (11). Vero cells, derived from African green monkey kidney cells, have been widely used in producing inactivated vaccines because of their high viral replication capacity and scalability (12). Nonetheless, concerns persist about potential contamination with non-human DNA, posing carcinogenic risks and complicating vaccine purification (13,14).

Human diploid cells, like the MRC-5 cell line derived from the lung tissue of a 14-week aborted Caucasian male fetus (15), offer a safer alternative. These cells have become increasingly popular for manufacturing viral vaccines, such as those for hepatitis A, varicella, and polio, and have proven susceptible to RSV (16). However, the MRC-5 cell line exhibits limitations, including reduced efficiency and genetic changes due to high passage numbers. This necessitates the development of an equivalent or superior cell line to maintain the efficacy and reliability of cell-based research and vaccine production.

Razi Vaccine and Serum Research Institute has established a new diploid cell line named RAZI-HDC (R-HDC), derived from the lung tissue of a 4-month-old female fetus. This study compares the RSV growth rate and proliferation in the R-HDC cell line with that in the MRC-5 cell line. The results indicate nearly identical growth rates and virus titers between the two cell lines, highlighting R-HDC's potential as a promising new platform for vaccine development and RSV research.

2. Materials and Methods

1. Cells :This study utilised three cell lines: Vero, MRC-5, and R-HDC. Vero cells were exclusively employed for viral titration. MRC-5 cells, susceptible to a wide range of viruses, are commonly utilised in producing viral vaccines. The R-HDC cell line based on our previous experiment regarding development of cell substrate from foreskin and dental pulp, derived from the lung tissue of an 18-week-old Iranian female fetus, was also sourced from RVSRI's Human Viral Vaccine Department (unpublished data) (17, 18).

2. Cell cultivation: MRC-5 cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM, Gibco BRL) supplemented with 8% fetal bovine serum (FBS, Gibco BRL) and kanamycin-neomycin (Biosera, UK). Cells were seeded at a density of 1×10^5 cells/ml in cell culture flasks (175 cm^2 or 25 cm^2) and incubated at 37°C. Subculturing occurred upon reaching confluence, approximately every four days, involving a wash with phosphate-buffered saline (PBS), followed by detachment using 0.025% trypsin. R-HDC cells followed the same protocol, with Subculturing adjusted to every five days.

3. Virus : The RSV Long strain, kindly provided by Louis Bont from Wilhelmina Children's Hospital, University Medical Center Utrecht, was employed in this study. Virus stocks were prepared in Vero cells using DMEM supplemented with 10% FBS and infected at a low multiplicity of infection (MOI, ~0.001). Viral cultures were harvested upon maximal cytopathic effect observation, typically between 3-4 days post-infection. The supernatant was collected, clarified by centrifugation, and stored at -80°C.

4. Cell Counting by Hemocytometer: Cell density was determined using a hemocytometer. Cells were trypsinisezd, suspended, and mixed with 0.4% Trypan blue in a clean microtube

(50µl each). The mixture was then counted under a microscope using an Improved Neubauer slide (HBG, Germany).

5. Cell Growth Kinetics: Growth kinetics were monitored by counting total cells in 25 cm^2 flasks at 24-hour intervals. This experiment utilised 24 flasks for either the MRC-5 or R-HDC cell lines, as previously described. The specific cell growth rate, µ (h^{-1}), and cell division number, Cd, were calculated using established methods by Samia Rourou.

6. Virus Titers in Terms of log_10TCID_50/mL: Vero cells, after detachment, were seeded in 96-well tissue culture plates using Minimum Essential Medium (MEM) with 10% FBS and 1× antibiotic-antimycotic solution. After reaching confluence by microscopically observation, cells were infected with tenfold serial dilutions of virus samples in MEM supplemented with 1.5% BSA. After four days at 37°C, cytopathic effects (cell rounding, detachment, and lysis) were visually assessed.

7. Infection of Cell Monolayer with RSV: Virus growth kinetics were determined using the methods described by Samia Rourou, employing the modified Koprowski method for virus inoculation. After removing the culture medium, the cell monolayer was washed with PBS, and the virus seed was applied in a minimal volume of DMEM. The monolayer was incubated for 45 minutes at 37°C, followed by further incubation in DMEM supplemented with 0.2% human albumin.

8. **Observation of Cytopathic Effect (CPE) in the Infected Cell Cultures:** CPE was monitored daily using an inverted microscope after virus inoculation. The supernatant from infected cells was inoculated onto a sensitive VERO cell line for further validation. After 72

hours at 37°C, CPE was assessed daily. Post-inoculation , VERO cells were fixed with 30% formalin and stained with crystal violet for enhanced visual confirmation.

9. Virus Growth Kinetics: To analyze virus growth kinetics, six-well plates with either R-HDC or MRC-5 cells at 90% confluence were inoculated in duplicates with a virus seed at a multiplicity of infection (MOI) of 0.01, using 500 µL of medium per well. Cell monolayers were harvested at predetermined times post-infection (6, 24, 48, 72, 96, and 120 hours), scraped into the supernatant, vortexed, snap frozen in liquid nitrogen, and stored at -80°C until analyzed by the TCID_50 assay. The specific virus production rate (P) was determined using the following equation (Eq. 1):

$$P = \frac{Cn+1-Cn}{\Delta t} \quad (1)$$

$C_n$and C_{n+1} represent the virus titers in focus-forming units per milliliter (*0.7 FFU/mL) (20) at consecutive time points t_n and t_n+1, respectively, and Δt is the time interval between these points. Overall virus productivity was calculated with the following equation (Eq. 2):

$$Overall\ Virus\ Productivity = \left(\frac{C}{Xinfection}\right) \times \left(\frac{1}{Xend} - \frac{1}{Xinfection}\right) \quad (2)$$

Here, $C\ C$ denotes the virus titer in CCID50/mL obtained at the end of the culture period, $X_{infection}$ is the cell density at the time of infection, and X_{end} is the cell density at the end of the culture period, with both densities expressed in cells/mL. The infection time refers to the duration of the virus production phase, expressed in hours.

10. Data Analyses

The data were analyzed using GraphPad Prism software and Microsoft Excel. The results represent three or more independent experiments and are shown as means with standard deviations. For comparisons between two groups with normally distributed data, either Student's two-tailed t-test or paired Student's one-tailed t-test were used.

3. Results

3.1. Cell proliferation

The growth dynamics of R-HDC cells were meticulously tracked under static culture conditions within 20Z flasks. Cell counts at successive time points were recorded, and the growth percentage was calculated by comparing the cell count at each time point to the maximum cell count observed during the growth period. These results are summarized in Table 1 and Diagram 1. During the initial eight days of cultivation, the cell counts and percentage growth (mean±SD (*10^5)) in the flasks were as follows: Day 1 - 12±0 (20%), Day 2 - 15.33±0.57 (53.33%), Day 3 - 21.33±2.51 (133.3%), Day 4 - 27±2.64 (170%), Day 5 - 34.6±2.08 (246.66%), Day 6 - 37.33±2.88 (273.33%), Day 7 - 41±1.73 (310%), and Day 8 - 41.66±2.08 (316.66%).

The growth rate analysis shows that R-HDC strain experienced a delayed growth phase from the 1st to the 3rd day after culture initiation. However, robust growth was observed until the fifth day during the logarithmic phase. Post the fifth day, the growth rate plateaued, matching that of the MRC-5 cell line. Based on these findings, passing R-HDC cells or using them for viral inoculation and vaccine preparation between the fifth and sixth days is recommended. Comparisons with the standard MRC-5 cell substrate showed no significant differences, suggesting that R-HDC cells are a suitable alternative for research and production.

The number of cell divisions at each time point was also calculated at 24-hour intervals. From the first to the eighth day of culture, the number of cell divisions recorded was 0.32±0.015,

0.51±0.01, 0.69±0.07, 0.83±0.07, 1.02±0.01, 1.17±0.11, 1.21±0.08, and 1.24±0.07, respectively. Most cell divisions occur by the seventh-day post-passage, indicating continuous cell division until this point. Statistical analysis showed no significant difference in cell division rates from the fifth to the eighth day ($p < 0.05$), suggesting that cell passaging or viral inoculation could be effectively performed during this period. Comparative analysis of cell division rates under static conditions for R-HDC and MRC-5 showed similar behaviors.

The specific growth rates were determined by averaging cell counts from three 20Z flasks at 24-hour intervals, as presented in Tables 5 and 6 (Figures 5 and 6). The specific growth rate of R-HDC cells on the eighth day was as follows: 0.0029 ± 0.00920, 0.006 ± 0.0123, 0.0083 ± 0.0108, and 0.0093 ± 0.0098, 0.010 and 0.011 ± 0.0003 μ (h−1). The specific growth rate was initially low and displayed a high standard deviation but gradually increased, reaching its peak during the fifth day. After this peak, the growth rate began to slow down. Comparative results of specific growth rates under static conditions showed that MRC-5 and R-HDC cells function similarly, achieving a dense monolayer by day 5, although the quality of calf serum can significantly impact this process (Table 1). The R-HDC cell growth curve exhibited a lag phase on the first day post-culture, followed by logarithmic growth until the day. Comparative analysis of the growth rates of MRC-5 and R-HDC over eight days showed that MRC-5 cells generally had a slightly higher growth rate, though differences were minimal and not statistically significant (Figure 1). The number of cell divisions (Cd) was calculated, revealing that the majority occurred on the eighth day post-passage, with no significant difference from day six to eight ($p < 0.05$) (Figure 2).

Table 1

Figure 1

Figure 2

The specific growth rate (μ) for R-HDC cells was lowest in the initial days of culture, increasing until peaking on day seven at an average of 0.012 before decreasing again. Conversely, the MRC-5 cells exhibited a rapid, specific growth rate from the onset, reaching a peak on day three at an average of 0.0135. However, no statistically significant difference in growth kinetics was observed between the two cell lines (Figure 3). In summary, R-HDC cells under static culture conditions initially showed a delayed growth phase, followed by a logarithmic increase until the fourth day and then a decline. Most cell divisions were noted on the eighth day post-passage. The specific growth rate of R-HDC cells gradually peaked on the fourth day. Although the growth rate was comparable to that of MRC-5, the slight differences observed were not statistically significant.

Figure 3

3. **Cell Infection Monitoring**

4. Both R-HDC and MRC-5 cell lines exhibited apparent cytopathic effects post-inoculation, characterized by cell rounding and detachment. This CPE was observable by the third-day post-inoculation and intensified over time (Figure 4. a.b.d.e). When the supernatant from the infected R-HDC cells was transferred to VERO cells, a pronounced CPE was also observed, confirming the infectivity and viability of viruses produced in R-HDC cells.

The VERO cells, post-inoculation, were fixed with 30% formalin and stained with crystal violet to enhance the visibility of the CPE, providing more apparent visual confirmation of the results (Figure 4. c.f).

Figure 4

Inoculation with RSV, showing clear signs of CPE, including rounding and detachment. C: VERO cell six days post-inoculation with virus produced in the R-HDC cell line, stained with crystal violet to highlight infected cells. D: Uninfected MRC-5 cells showing no signs of cytopathic effect. E: MRC-5 cells six days post-inoculation with RSV, showing clear signs of CPE, including rounding and detachment. F: VERO cells six days post-inoculation with virus produced in the MRC-5 cell line, stained with crystal violet to highlight infected cells.

4. **Virus Growth Kinetics**

To assess the impact of cell substrate on virus productivity, numerous flasks were prepared and inoculated with virus seeds at an MOI of 1/10 and incubated using the monolayer method. As our previous experiment cell concentrations and virus titers were measured at 24-hour intervals, with the specific virus production rate and overall virus productivity calculated from these data (Table 2) (17, 18).

Virus titers in MRC-5 and R-HDC cell lines consistently increased throughout the incubation period, peaking between the third and fourth days. Specifically, the specific virus production rate for MRC-5 cells was initially 0.001 ± 0.0035 CCID50 per cell per hour on day 1, escalating to 3.3 ± 0.0009 CCID50 per cell per hour by day 4. Similarly, R-HDC cells exhibited a specific virus production rate of 0.00092 ± 0.27 CCID50 per cell per hour on day 1, increasing to 1.15 ± 0.14 CCID50 per hour by day 3 (Figure 5). Both cell lines experienced a significant reduction in cell density over the six-day viral replication period, with cell lysis or rounding and detachment peaking on day 4. The maximum viral productivity was observed at 2.5 for MRC-5

and 2.468571 for R-HDC before a noted decrease in both cell lines, particularly in MRC-5 (Table 2).

Figure 5

Table 2

These values demonstrate the dynamics of virus replication and cell density reduction over time, providing insights into the productivity and efficiency of virus production. The virus growth medium was DMEM supplemented with 0.2% human albumin. Specific virus production rates for MRC-5 (a) and R-HDC (b) and virus productivity for MRC-5 (c) and R-HDC (d) are depicted. This section delineates the dynamics of viral replication and cytopathic effects in MRC-5 and R-HDC cells.

4. Discussion

This article evaluates the potential of the R-HDC cell line as a new cell substrate for producing RSV vaccine candidates. RSV is a predominant pathogen responsible for severe respiratory infections in both young children and older adults, contributing substantially to global morbidity and mortality. The study contrasts RSV growth in R-HDC cells with the well-established MRC-5 cell line, addressing the critical need for a safe and effective vaccine against a virus that imposes a significant healthcare burden worldwide. The relevance of this study stems from the urgent requirement to develop a reliable vaccine for RSV, a leading cause of respiratory infections in vulnerable demographics, including infants and the elderly (17). In order to better understand the behavior of the RS virus in terms of growth in a new cell substrate and to determine the optimal conditions for virus replication for production or research purposes, it is necessary to determine the growth kinetics of the virus in that cell. In order to investigate the kinetics of the growth and

multiplication of RSV on the R-HDC cell substrate, various factors must be measured and monitored. The most important of these factors, which include the investigation of the sensitivity of R-HDC cells to RSV, adaptation of A2 strain RSV virus on R-HDC cells and the increase of virus titer, were measured in the laboratory. The findings of the study from the evaluation of the growth of the respiratory syncytial virus on the new cell substrate have shown that the kinetics of the virus growth is satisfactory compared to the standard cell line MRC-5. Keeping the virus titer and increasing it is one of the most critical vital findings necessary for the continuous production of the vaccine, which was confirmed through a comparative analysis with the standard cell. The examination of growth kinetics demonstrated R-HDC's viability as a substrate for viral vaccine production, with growth patterns comparable to those of MRC-5 cells. The study further investigates the kinetics of virus growth and productivity of both cell lines following RSV inoculation. Results indicated stable virus titers and comparable specific virus production rates between R-HDC and MRC-5 cells, supporting the efficient replication of RSV within R-HDC cells. These observations provide critical insights into the dynamics of virus replication and cell density reduction over time, parameters vital for evaluating the efficiency of vaccine production.

5. Conclusion

The findings suggest that R-HDC cells are promising cell substrate for RSV vaccine production. The cell line's performance, comparable to that of MRC-5 cells in supporting virus growth, underscores its potential as an alternative platform for vaccine development. Future research should optimize culture conditions and evaluate the immunogenicity and efficacy of vaccines produced using the R-HDC cell line in pre-clinical and clinical settings. In conclusion, this study underscores the potential of the R-HDC cell line as a new cell substrate for RSV vaccine production. By demonstrating its compatibility and efficacy in supporting RSV propagation, the

study opens avenues for further exploration of R-HDC as a safe and reliable substrate for research and development. The results significantly contribute to the ongoing efforts to mitigate RSV's substantial public health impact through effective vaccine strategies.

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Authors' Contribution

1- Study concept and design: Ashraf Mohammadi & Vahid Salimi
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Author Disclosures

The article we have submitted to the journal for review is original, written by the stated authors, and not published elsewhere.

Ethics: No animal used in this project

Conflict of Interest

The authors declare that they have no conflict of interest.

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Data availability

The required data are available from the corresponding author upon reasonable request.

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Table 1. Growth and Cell Division Metrics of MRC-5 and R-HDC Cells Over Time

Sampling time (hours after passage)	Total Cell Count Per 25 Cm2 Flask ($*10^5$) Mean ± SD		% Growth Mean ± SD		Cell Division Number(Cd) Mean ± SD	
	MRC-5	R-HDC	MRC-5	R-HDC	MRC-5	R-HDC
0	10	10	0.0	0.0	0	0
24	12.0 ± 1.0	12±0	26.7±2.2	20±0	0.36±0.03	0.32±0.015
48	15.6 ± 2.1	15.33±0.57	34.8±4.6	53.33±5.77	0.47±0.06	0.51±0.01
72	24.3 ± 2.5	21.33±2.51	54.1±5.6	133.3±25.16	0.73±0.08	0.69±0.07
96	29.6 ± 2.5	27±2.64	196.6±±1.9	170±26.45	0.89±0.08	0.83±0.07
120	36.6 ± 3.7	34.6±2.08	266.6±37.8	246.66±20.81	1.10±0.11	1.02±0.01
144	41.0 ± 4.5	37.33±2.88	310±45.8	273.33±28.86	1.23±0.14	1.17±0.11
168	43.3 ± 2.3	41±1.73	333.3±23	310±17.32	1.30±0.07	1.21±0.08
192	44.0 ± 2.6	41.66±2.08	340±26.45	316.66±20.81	1.32±0.08	1.24±0.07

٣٧٥ **Table 2: Data on virus titer, total cell number, specific virus production rate, and virus productivity at**

٣٧٦ **different time points post-virus inoculation for MRC-5 and R-HDC cells**

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Time After Virus Inoculation	Mean ±SD of Virus Titer in Harvest CCID/50)/ml		Total Cell Count Per/Ml Mean ± SD		Specific Virus Production Rate		Virus Productivity	
Hrs	MRC-5	R-HDC	410000		CCID50/Cell/H		Mean ± SD	
			MRC-5	R-HDC	MRC-5	**R-HDC**	MRC-5	**R-HDC**
24	$3.4*10^2$	$3.23*10^2$	$400±100*10^3$	$400±125*10^3$	0.001± 0.0035	0.00092 ± 0.27	0.01394	0.0132
48	$4*10^3$	$3.9*10^3$	$420±40.4*10^3$	$405±41*10^3$	0.08±0.0027	0.11±0.14	0.0032	0.156
72	$4.66*10^4$	$1.527*10^5$	$411±95*10^3$	$400±51.2*10^3$	2.440.0014	1.15±0.14	1.8676	0.2107
96	$5*10^5$	$4.32*10^5$	$390±76.3*10^3$	$396±100*10^3$	3.3±0.0009	1.04±0	2.5	2.4685
120	$5.66*10^5$	$6.07*10^5$	$350± 3.2*10^3$	$396±147.3*10^3$	1.65±0.0008	0.11±0.25	0.159	0.3602
144	$5*10^5$	$5.05*10^5$	$310± 1.1*10^3$	$380±0.1*10^3$	0.52±0.0008	0.13±0.25	0.0345	0.1161