Original Article

Sensitivity of a Novel Lizard-Derived Cell Line (Z1) to Measles, Rubella and Respiratory Syncytial Viruses

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ABSTRACT

The objective of this study was to assess the sensitivity of the Z1 cell line to measles, rubella, and respiratory syncytial viruses (RSVs), and to observe the subsequent cytopathic effects (CPEs) that result from these viruses. The study sought to determine the potential of the Z1 cell line as a suitable alternative for more efficient isolation and identification of these viruses, as well as for conducting quality control tests, such as potency tests and the production of biological products, including diagnostic antigens. The Z1 cell lines were prepared in disposable cell culture flasks and were inoculated with Measles, Rubella, and Respiratory Syncytial Viruses at defined multiplicity of infection. Vero-WHO, Rabbit Kidney-13 (RK-13), and Hep-2 cell lines were also prepared as standard cell substrates and were inoculated with measles, rubella, and respiratory syncytial viruses, respectively. The quality and quantity of CPE formation, i.e., the microscopic signs of viral replication, were observed daily in the test and control cell lines. Samples were collected for the purpose of calculating the titer of progeny viruses, which was then used to compare the viral yield. An interference test using the Vesicular Stomatitis Virus (VSV) was also performed to confirm the replication of Rubella in the Rubella-inoculated cell cultures. In the case of Measles Virus, a significant difference was observed between viral yield in Z1 and Vero-WHO cells. A similar outcome was observed for the Rubella virus, with significant disparities in viral yield observed between Z1 and RK-13 cells. However, when Respiratory Syncytial Virus was inoculated into the Z1 and Vero-WHO cells, no significant differences in viral yield were observed. Consequently, the Z1 cell line emerges as a promising substitute for Hep-2 cells, particularly for the isolation and propagation of Respiratory Syncytial Virus. However, it should be noted that the Z1 cell line exhibits a higher susceptibility to Measles Virus. However, the Z1 cell line was not found to be a suitable substrate for the propagation of Rubella Virus.

Keywords: Z1, Propagation, Measles, Rubella, RSV, Permissive, Cell line.

1. Introduction

Significant advancements have been made in the field of virology since the inception of cell culture techniques for propagating vaccinia viruses in 1913. This was followed by the propagation of yellow fever and smallpox viruses in the 1930s (1-3). The advent of cell lines permissive to numerous viruses marked a pivotal shift in the field of virology, thereby enabling the in vitro cultivation of a multitude of viruses. This method was significantly more convenient than traditional methods, which relied on eggs and experimental animals. Consequently, cell culture became the prevailing standard method for the isolation, identification, and diagnosis of numerous viruses (2). The advent of cell culture techniques led to a significant shift, wherein laboratory animals were increasingly replaced by cell cultures in numerous experimental settings (1). In the contemporary era, cell cultures have assumed a pivotal role in various domains, including the isolation and identification of viruses, the diagnosis of viral infections, the production of biological products, including vaccines, and the execution of quality control testing. The continuous development of new sensitive cell lines is of crucial importance in the field of virology research. Those cell lines that support viral replication, particularly with a distinct cytopathic effect (CPE), could be considered as invaluable tools in both basic and clinical virology research. This study focuses on the Z1 cell line, a novel development that has been successfully characterized and patented (4) by researchers at the Razi Vaccine and Serum Research Institute (RVSRI) from a lizard's tail (Cyrtopodion scabrum) in 2010. Following this, the cell line was formally registered and subsequently stored within the biobank of the RVSRI. The present study aims to assess the sensitivity of this cell line to measles virus (MV), rubella virus (RV), and respiratory syncytial virus (RSV).

2. Materials and Methods

2.1. Viruses and Cell Lines

2.1.1. Measles Virus

The vaccine strain of measles virus, designated AIK-C, was provided by the QC department of RVSRI.

2.1.2. Rubella Viru

The vaccine strain of the rubella virus, designated Takahashi, was provided by the QC department of RVSRI.

2.1.3. Respiratory Syncytial Virus

RSV was provided by the virology department, Faculty of Public Health, Tehran University of Medical Sciences (TUMS).

2.1.4. Vero-WHO

The QC department at RVSRI provided the control cell substrate (against Z1) for the inoculation of the MV.

2.1.5. RK-13

The control cell substrate (against Z1) for the inoculation of the RV was supplied by the QC department at RVSRI.

2.1.6. Hep-2

The virology department at TUMS provided the control cell substrate (against Z1) for the inoculation of RSV.

2.1.7. Z1

The test cell substrate was used for the inoculation of the MV, RV and RSV, which were provided by the QC department at RVSRI.

2.2. Cell Culture Preparation

Cell cultures were prepared in 25 and 75 cm flasks using DMEM (Dulbecco's Modified Eagle Medium) supplemented with 5% Fetal Bovine Serum (FBS, foetal bovine serum). The flasks were then placed in an incubator set to 37°C and containing 5% CO2 until confluent cell monolayers had formed, at which point the virus inoculation was performed.

2.3. Cell Counting

In order to inoculate the cell cultures at a defined multiplicity of infection (MOI), it is first necessary to calculate the viable cell population in the flask. The Vero-WHO, RK-13, Hep-2, and Z1 cell lines were prepared as monolayers, then trypsinised, stained with trypan blue dye, and counted using a Neubauer chamber (5).

2.4. Virus Titration

The microtitration process was conducted in cell culture microplates. 10-fold serial dilutions (10-1-10-9) were prepared and each dilution was inoculated into four wells. The appearance of the CPEs was meticulously monitored on a daily basis for a period of seven days. The Reed and Muench method was used to calculate the viral titer in the MV, RV, and RSV stocks and harvested material (6).

2.5. Virus Inoculation

MV, RV, and RSV were inoculated into the prepared cell monolayers at an MOI of 0.03, 0.03, and 0.05, respectively.

2.6. Evaluation of CPEs

The inoculated cell monolayers were observed on a daily basis. Any progress in the quality and quantity of CPEs was meticulously documented. Monolayers inoculated with MV-, RV-, and RSV were observed until day 7, 7, and 3 post-inoculation, respectively, at which time the highest extent of CPE typically manifests in the relevant control cell substrates. Samples for titration were also collected concurrently.

2.7. The procedure for Inoculation of Viruses into Control and Test Cell Lines

The inoculation of MV into Z1 and Vero-WHO was carried out as follows: Z1 and Vero-WHO cell cultures were inoculated at the defined multiplicity of infection (MOI). The formation of CPEs was meticulously observed and documented on a daily basis. Samples were collected from the cell culture media when the inoculated cell cultures exhibited the maximum CPEs. Subsequently, a comparison was made between the viral titers present in the harvested materials from the two cell substrates. To ascertain the reproducibility of the experiment, it was repeated on three separate occasions. The inoculation of RV into Z1 and RK-13 cell lines followed the same procedure. Inoculation of RSV into Z1 and Hep-2: The same procedure was repeated, this time using Z1 and Hep-2 cell lines.

2.8. Interference Test

Inoculation of RV to permissive cell lines does not necessarily result in the formation of distinct CPEs. The interference test has been developed as a means to assess RV proliferation in the absence of CPEs. To this end, RV-inoculated cell cultures are re-inoculated with VSV after 72-96 hours, in the absence of CPEs. The observation of CPE (cell lysis) after 24-48 hours indicates that the cell substrate is not permissive to RV. Conversely, the absence of cell lysis demonstrates that RV has successfully replicated within the cell substrate, despite the absence of CPE.

2.9. Data Analysis

The qualitative evaluation of CPE formation following inoculation of the viruses was conducted. The viral titers present in the harvested materials were then subjected to analysis using SAS 9.4, employing the probit procedure. Multiple comparisons of titers derived from different cell types were conducted using the Tukey test.

3. Results

3.1. Inoculation of MV to Z1 and Vero-WHO Cell Lines

The formation of CPEs, as indicated by the development of syncytia, manifested 24 hours post-inoculation, reaching a peak on day 7 post-inoculation (Figure 1). The mean of the MV titer in the samples from inoculated Vero-WHO cells was 10 times higher than that of the Z1-inoculated ones. The comprehensive data concerning the extent of CPE formation and the MV titer in harvested materials from three inoculation runs are outlined in Table 1. A statistically significant difference was observed between the MV titer in the harvested materials from the test and control cell substrates ($P < 0.05$) (Table 2, Figure 2).

3.2. Inoculation of RV to Z1 and RK-13 Cell ines

Following inoculation of RV, CPEs manifest as clamp-shaped views, though they are typically not readily perceptible. Their emergence occurs 48 hours post-inoculation, reaching a maximum on day 7 (see Figure 3). The comprehensive data concerning the extent of CPE formation from three inoculation runs have been summarized in Table 3. Notably, no CPEs were observed in Z1 cells even at day 7 post-inoculation, prompting the execution of an interference test using VSV. The RV-inoculated RK-13 cells exhibited attachment to the flask, while the RV-inoculated Z1 cells demonstrated complete detachment from the flask, resulting from VSV replication (Figure 3). This outcome serves to underscore the incompatibility of Z1 cells with RV. The RV titer in the samples from RV-inoculated RK-13 and Z1 cells during three inoculation runs is summarized in Table 3. A statistically significant difference was observed between the RV titer in the harvested materials from the test and control cell substrates ($P<0.05$).

3.3. Inoculation of RSV to Z1 and Hep-2 Cell Lines

CPEs, manifesting as cell rounding, emerged 24 hours post-inoculation, reaching a peak on day 3 post-inoculation (Figure 4). The detailed data concerning the extent of CPE formation and RSV titer in harvested materials during three inoculation runs are summarized in Table 4. The RSV titer in the harvested materials from the test and control cell substrates did not differ significantly ($P<0.05$) (Table 2, Figure 5).

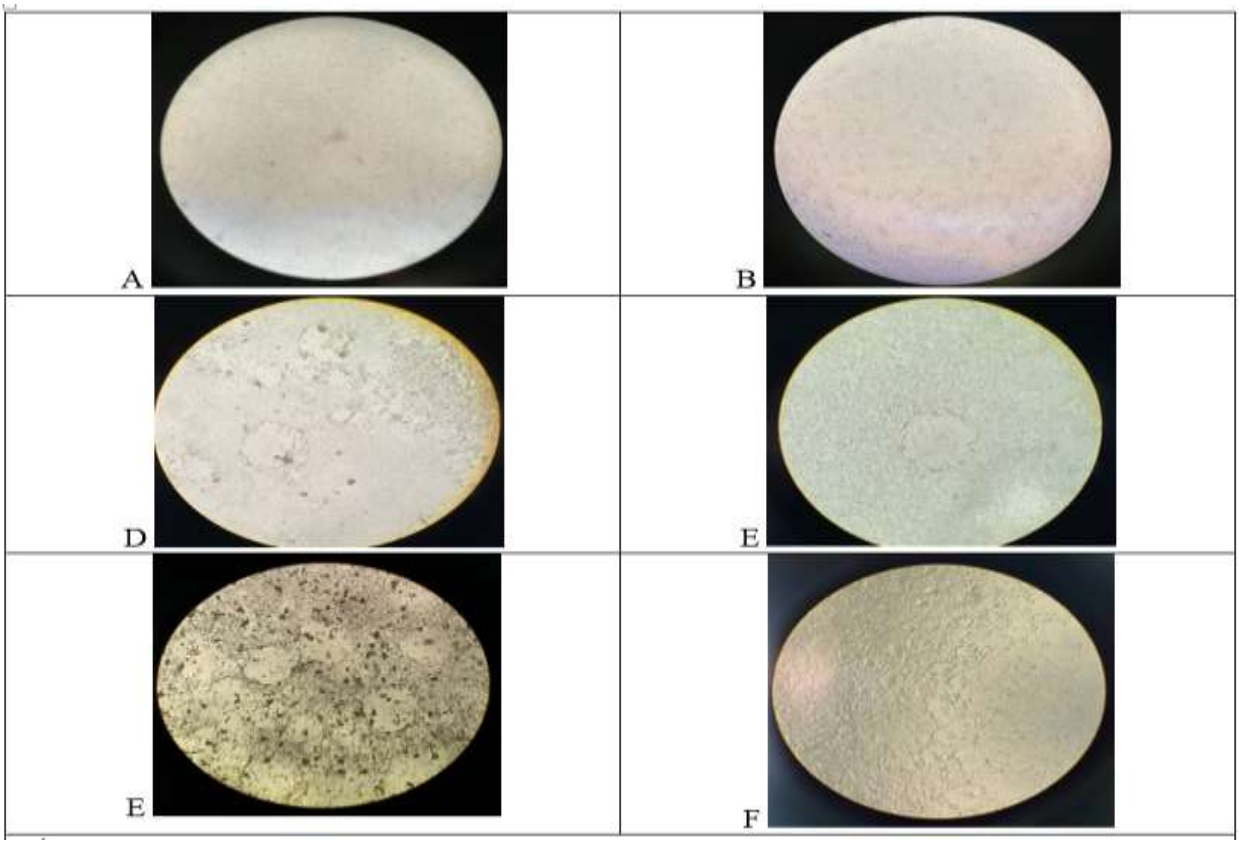

Figure 1: Left: un-inoculated Vero-H cell substrate (10X), Right: un-inoculated Z1 cell substrate (10X).

Table 1. The extent of induced CPE and the viral titer (CCID50) in harvested materials on day 7 post-inoculation. MV: Measles virus.

Order of runs	Vero-WHO cell culture (control)		Z1 cell culture (test)	
	extent of CPE	MV titer	extent of CPE	MV titer
1	80%	$10^{4.25}$/ml	70%	$10^{3.75}$/ml
2	90%	$10^{4.50}$/ml	60%	$10^{3.50}$/ml
3	100%	$10^{4.75}$/ml	50%	$10^{3.25}$/ml
Mean	90%	$10^{4.50}$/ml	60%	$10^{3.50}$/ml

Table 2. Statistical analysis of the viral titers. (Log10 $CCID_{50}$ with a 95% confidence interval).

Virus	Rep	Cell		P. Value
		Control cell	Z1cell	
Measles	1	4.27 (3.18-5.2367)	3.73 (2.76-4.81)	
	2	4.50 (3.49-5.50)	3.50 (2.38-4.61)	
	3	4.76 (3.73-5.76)	3.27 (2.18-4.23)	
	Overall	4.52 (4.06-4.97)	3.50 (3.09-3.90)	0.018
Rubella	1	7.49 (6.50-8.99)	0.00	
	2	7.23 (6.27-8.39)	0.00	
	3	7.73 (6.73-8.97)	0.00	
	Overall	7.48 (7.02-8.00)	0.00	<0.01
RSV	1	5.46 (4.50-6.51)	4.73 (3.76-5.81)	
	2	5.50 (4.50-6.50)	5.27 (4.19-6.24)	
	3	5.50 (4.39-6.61)	5.00 (4.01-5.99)	
	Overall	5.48 (5.04-5.93)	5.00 (4.57-5.43)	0.66

Note: Significant difference is considered at P<0.05.
Vero-H cells are used as Control cell substrate of Measles virus, RK-13 cells are used as Control cell substrate of Rubella virus, and Hep-2 cells are used as Control cell substrate of Respiratory Syncytial virus. Significant differences.

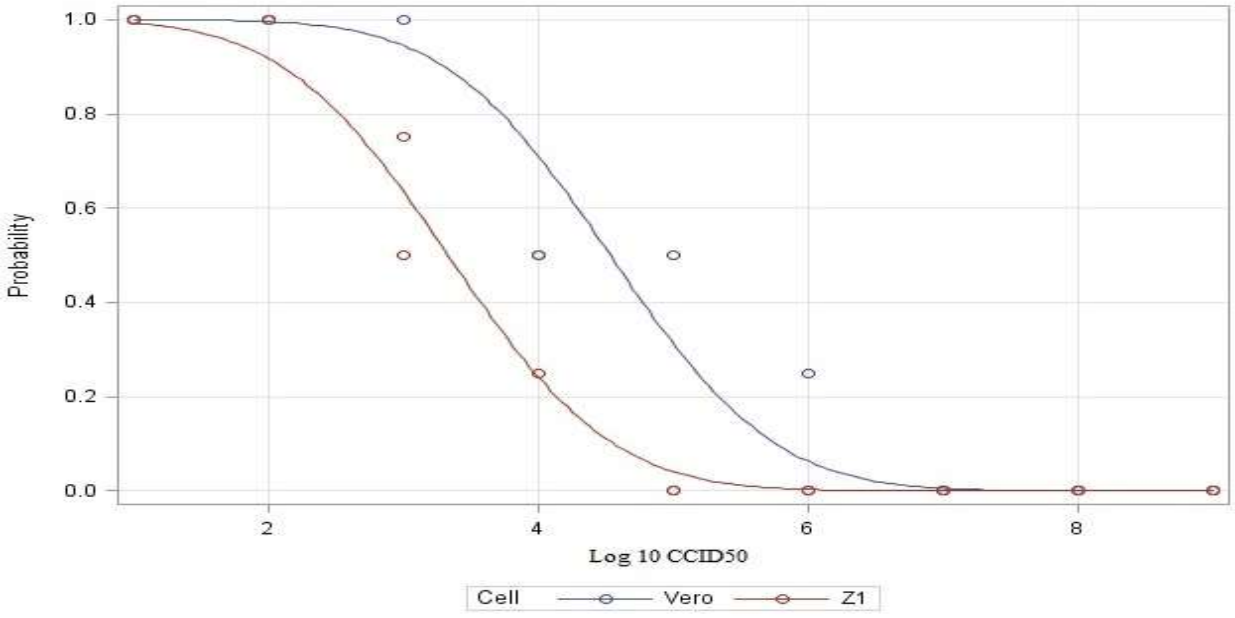

Figure 2. Comparison of titration (Log10 $CCID_{50}$ and 95% confidence interval) of Measles virus on Vero and Z1 cells.

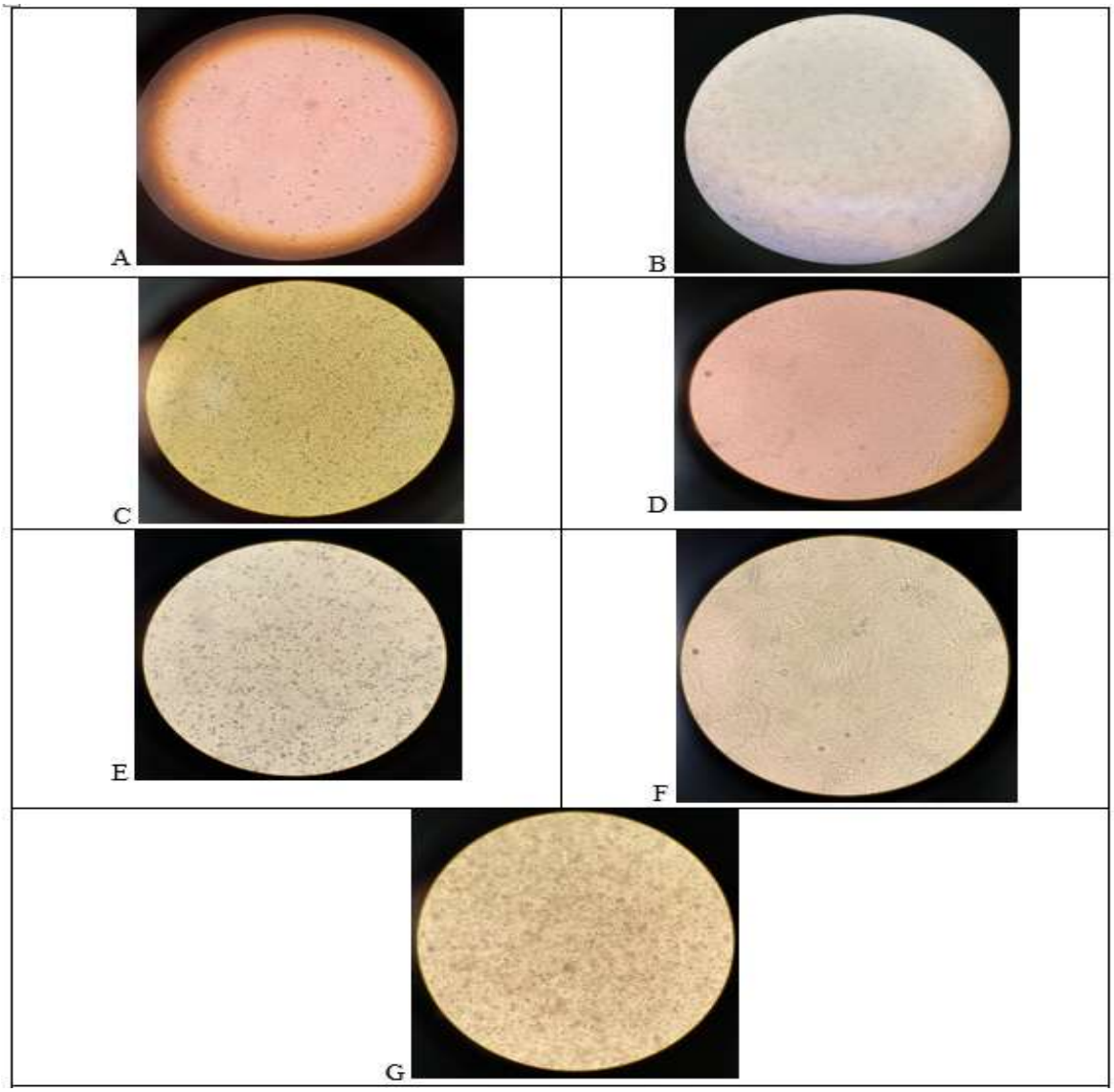

Figure 3. A) Un-inoculated RK-13 cell substrate (10X), **B)** un-inoculated Z1 cell substrate (10X), **C)** RV-inoculated RK-13 cell substrate (day 4, 10X), **D)** RV-inoculated Z1 cell substrate (day 4, 10X), **E)** RV-inoculated RK-13 cell substrate (day 7, 10X), **F)** RV-inoculated Z1 cell substrate (day 7, 20X), **G)** Interference test on RK-13 cell substrate, day 7 post-inoculation of RV (10X).
RV: Rubella Virus.

Table 3. The extent of induced CPE and the viral titer (CCID50) in harvested materials on day 7 post-inoculation.
RV: Rubella Virus.

Order of runs	RK-13 cell culture (control)		Z1 cell culture (test)	
	extent of CPE	RV titer	extent of CPE	RV titer
1	60%	$10^{7.50}$/ml	0%	Not detected
2	50%	$10^{7.25}$/ml	0%	Not detected
3	70%	$10^{7.75}$/ml	0%	Not detected
Mean	60%	$10^{7.50}$/ml	0%	Not detected

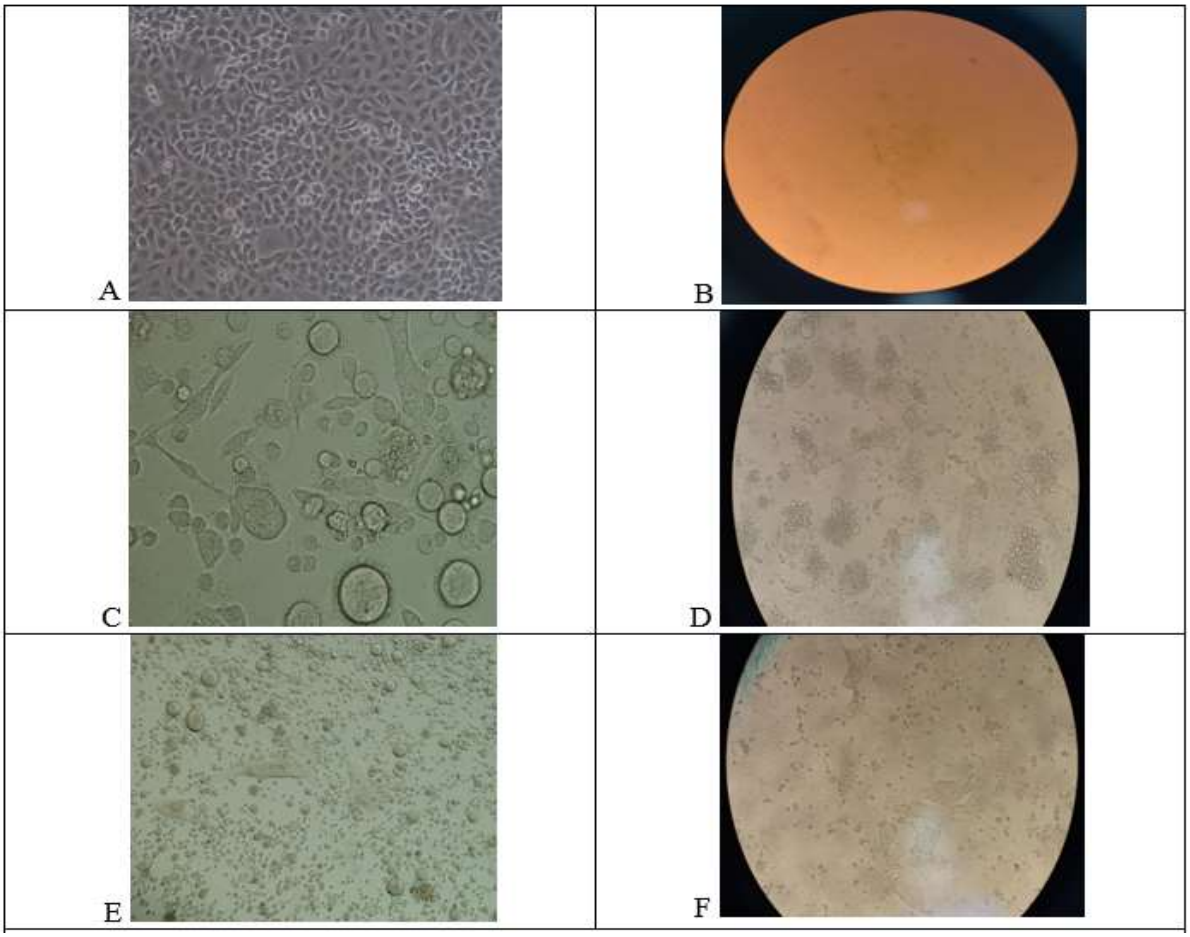

Figure 4: A) un-inoculated Hep-2 cell substrate (20X), **B)** un-inoculated Z1 cell substrate (10X), **C)** RSV-inoculated Hep-2 cell substrate (day 2, 40X), **D)** RV-inoculated Z1 cell substrate (day 2, 20X), **E)** RSV-inoculated Hep-2 cell substrate (day 3, 10X), **F)** V-inoculated Z1 cell substrate (day 3, 10X). RSV: Respiratory Syncytial Virus.

Table 4. The extent of induced CPE and the viral titer (CCID50) in harvested materials on day 7 post-inoculation. RSV: Respiratory Syncytial Virus.

Order of runs	Hep-2 cell culture (control)		Z1 cell culture (test)	
	extent of CPE	RSV titer	extent of CPE	RSV titer
1	90%	$10^{6.00}$/ml	50%	$10^{4.75}$/ml
2	90%	$10^{6.00}$/ml	70%	$10^{5.25}$/ml
3	90%	$10^{6.00}$/ml	60%	$10^{5.00}$/ml
Mean	90%	$10^{6.00}$/ml	60%	$10^{5.00}$/ml

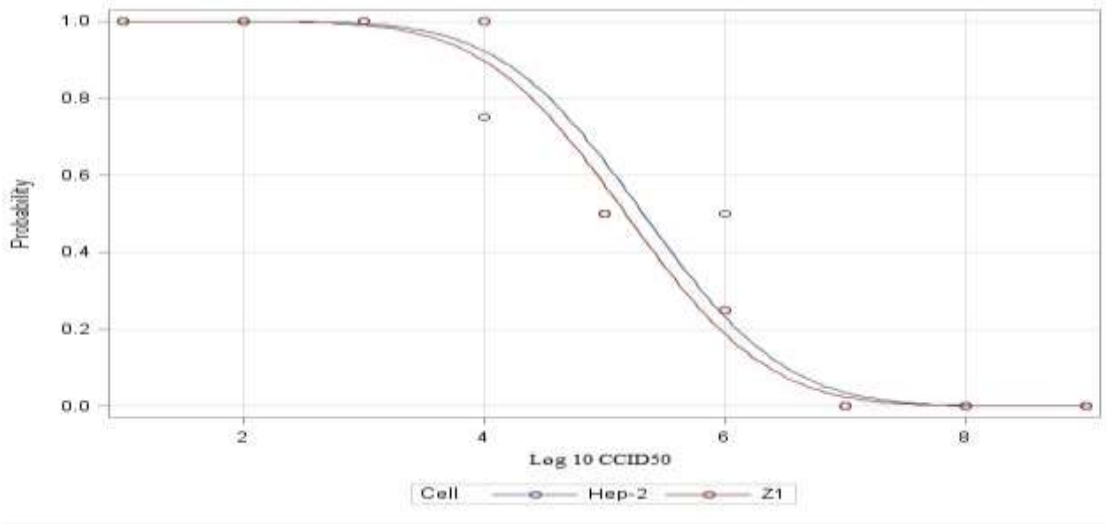

Figure 5. Comparison of titration (Log10 $CCID_{50}$ and 95% confidence interval) of Respiratory Syncytial Virus on Vero and Z1 cells.

4. Discussion

As obligate intracellular organisms, viruses could only be replicated in viable cells. Since the advent of cell culture techniques for viral inoculation approximately one hundred years ago, significant advancements have been made in the field of virology. In the present age, the utilization of cell cultures has expanded beyond the mere isolation, identification, and propagation of viruses. They now play a pivotal role in the diagnosis of numerous viral diseases and the production of a wide array of biological products, including viral vaccines. In recent decades, a diverse array of cell lines has been developed from a wide variety of species, including humans, animals, and plants. A primary focus of research in this field involves the evaluation of these cell lines' sensitivity to various viruses and their capacity to support viral replication. The permissiveness of a cell line to a broad spectrum of viruses, its capacity to support viral replication, and its propensity to form distinct CPEs are considered hallmark characteristics of a newly developed cell line. Reptile virology, a relatively recent field of study, has undergone rapid development in recent decades. The infection of reptiles with members of various viral families, including Adenoviridae, Herpesviridae, Paramyxoviridae, and Reoviridae, has been well documented (7). Consequently, the permissiveness of lizard-derived cells against multiple human and animal viruses can be predicted. The Z1 cell line, a notable achievement, was successfully developed by researchers at RVSRI. This cell line originates from the tail of the Cyrtopodian scabrum lizard. This cell line has been shown to be amenable to easy passage and, intriguingly, demonstrates a tolerance for temperatures ranging from 30 to 45°C in the presence of 5% CO2 in an incubator. The sensitivity of this cell line to certain viruses was assessed; however, the results have not been published in a peer-reviewed journal. The present study aims to investigate the sensitivity of this cell line against MV, RV, and RSV. A cell line with the same origin was established and characterized by another Iranian team, and the results were published in 2018 (8). The current study reports no further data on this cell line. In this research, as the first systematic study on the permissiveness of the Z1 cell line, several considerations were taken into account to select the viruses for inoculation experiments. Firstly, MV and RV vaccine strains have been utilized in MMR vaccine production for decades, and their safety has been thoroughly documented. These viruses are capable of culturing under normal virology laboratory conditions, with no biosafety concerns. Inoculation of RSV to cell cultures was performed at the virology department of TUMS, where RSV handling under biosafety level 2 has been done for years. Secondly, the RVSRI and TUMS laboratories possess the necessary skills and expertise to cultivate selected viruses under optimal conditions. Thirdly, standard cell substrates as control cell lines (Vero-WHO, RK-13, and Hep-2) against the under study cell line (Z1) were available. Additionally, characterized MV, RV, and RSV were readily available at RVSRI and TUMS. The optimal cultivation conditions for these viruses, including the multiplicity of infection (MOI), were defined in this study. These conditions were determined based on previous experiences and written procedures relevant to the selected cell lines and virus strains. The extent of CPE was evaluated using a qualitative method (microscopic observation), while the viral titer was assessed through a semi-quantitative approach (CCID50/ml). To ensure the validity of the acquired data, all experiments were performed three times. A review of the extant literature revealed the development of numerous cell lines from diverse reptile species over the past decade. However, a paucity of data regarding the evaluation of these cell lines' permissiveness to viruses was identified. Conversely, there is a paucity of literature addressing the permissiveness of the Z1 and analogous cell lines to viruses. Consequently, a direct comparison between the

findings of the present study and those of related studies was not feasible. However, the acquired data from this research could be interpreted on its own. The primary objective of this research was to evaluate the potential of the Z1 cell line for successful support of RSV, RV, and MHV replication, which may result in its application for virus isolation, diagnosis, and the production of biological products, among other things. The results of this study indicate that the Z1 cell line is a suitable cell substrate for successful propagation of RSV. While the yield of RSV is lower in comparison to Hep-2, the Z1 cell line facilitates expeditious viral replication and the formation of distinct CPEs. Further optimization runs may enhance the yield, thereby rendering the Z1 cell line more applicable for RSV propagation. In the context of MVA, the Z1 cell line facilitates its replication under sub-optimal conditions. Given the sensitivity of highly specialized cell lines, such as Vero-SLAM, the employment of the Z1 cell line in MV-related research appears to be an illogical approach. It is noteworthy that the Z1 cell line does not support replication of RV. The precise receptor of RV remains to be delineated; however, myelin oligodendrocyte glycoprotein (MOG), a constituent of the immunoglobulin superfamily, has been posited as a potential receptor of RV (9). The potential for RV to enter but not replicate within the Z1 cell line, if it lacks receptors, should be determined through molecular studies. If non-permissiveness is due to a receptor deficiency, it could be interpreted that the Z1 cell line most probably does not support other viruses that use the same receptor.

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Authors' Contribution
KSH and ML conceptualized and designed the study, reviewed and revised the initial manuscript which had been drafted by MKSH. MMN invented the Z1 cell line. VS supervised assessment of Z1 against RSV at TUMS. ARY analyzed the data. All authors approved the final manuscript as submitted and agreed to be accountable for all aspects of the work.

Ethics
The present study did not involve any human or animal subjects or samples, thus precluding any ethical concerns. However, ethical approval for conducting the study was granted by the Ethics Committee of Razi Vaccine and Serum Research Institute.

Conflict of Interest
The authors have declared that there is no conflict of interest regarding the present study.

Funding Statement
This study was supported by Razi Vaccine and Serum Research Institute.

Data Availability
All data generated or analyzed during this study are included in this article and its supplementary material files. Inquiries regarding the study's findings or methodology can be directed to the corresponding author.

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