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

Running title: Sensitivity of Z1cell line to Measles, Rubella and Respiratory Syncytial Viruses.

Abstract

This study aimed to investigate the sensitivity of the Z1 cell line to Measles, Rubella and Respiratory Syncytial Viruses, and to follow up on the formation of subsequent cytopathic effects for evaluating the potential of the Z1 cell line as a suitable alternative for easier isolation, and identification of these viruses, and even quality control tests such as potency tests and production of biological products such as 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 as the microscopic signs of viral replication in the test and control cell lines were daily observed. Samples were taken and the titer of progeny viruses was calculated to compare the viral yield. An interference test using the Vesicular Stomatitis Virus was also performed to confirm the replication of Rubella in the Rubella-inoculated cell cultures. In the case of Measles Virus there was a significant difference between viral yield in Z1 and Vero-WHO cells. For Rubella Virus there was also a significant differences in viral yield in Z1 and RK-13 cells. Despite these, there were no significant difference between viral yield, when Respiratory Syncytial Virus inoculated to the Z1 and Vero-WHO cells. Collectively, the Z1 cell line could be considered a suitable alternative to Hep-2 for isolation and propagation of Respiratory Syncytial Virus, while it is relatively sensitive

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to Measles Virus. Despite this, the Z1 cell line could not be used as a cell substrate for Rubella Virus propagation.

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

1. Introduction:

Significant progress in virology has been made since cell culture was for the first time used to propagate vaccinia viruses in 1913, followed by the propagation of yellow fever and smallpox viruses in the 1930s (1-3). The introduction of cell lines permissive to many viruses was a turning point in virology and made growing many viruses in vitro possible. It was much more convenient to use cell cultures in comparison to eggs and experimental animals. It gradually became a gold standard method of isolation and identification of many viruses and diagnosis of viral infections (2). Thanks to the discovery of cell culture techniques, laboratory animals were replaced with cell cultures in many experiments (1). Nowadays, cell cultures have a special place in the isolation and identification of viruses, diagnosis of viral infections, production of biological products including vaccines, and quality control testing, as well.

The continuous development of new sensitive cell lines plays a crucial role in virology researches. 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 was focused on the Z1, a novel cell line that has been successfully developed, characterized, and patented (4) by researchers at the Razi Vaccine and Serum Research Institute (RVSRI) from a lizard's tail (Cyrtopodion scabrum) in 2010. This cell line then was registered and banked at RVSRI biobank. In this research, the sensitivity of this cell line to measles virus (MV), Rubella virus (RV), and Respiratory Syncytial Virus (RSV) was assessed.

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2. Material and Methods:

2.1. Viruses and cell lines:

Measles Virus: AIK-C, a vaccine strain of the Measles virus, was provided by the QC department, RVSRI.

Rubella Virus: Takahashi, a vaccine strain of the Rubella virus, was provided by the QC department, RVSRI.

Respiratory Syncytial Virus: RSV was provided by the virology department, Faculty of public health, Tehran University of Medical Sciences (TUMS).

Vero-WHO: As the control cell substrate (against Z1) for inoculation of the MV was provided by the QC department, RVSRI.

RK-13: As the control cell substrate (against Z1) for inoculation of the RV was provided by the QC department, RVSRI.

Hep-2: As the control cell substrate (against Z1) for inoculation of the RSV was provided by the virology department, TUMS.

Z1: As the test cell substrate for inoculation of the MV, RV and RSV provided by the QC department, RVSRI.

2.2. Cell culture preparation:

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Cell cultures were prepared in 25 and 75 cm flasks using DMEM plus 5% Fetal Bovine Serum (FBS). The flasks were incubated at 37°C incubator containing 5% CO_2 until the formation of confluent cell monolayers when they got ready for the virus inoculation.

2.3. Cell counting:

To inoculate the cell cultures at a defined multiplicity of infection (MOI), the viable cell population in the flask should be calculated. Vero-WHO, RK-13, Hep-2, and Z1 cell lines were separately prepared as monolayers, then trypsinized, stained with trypan blue dye, and counted using a Neubauer chamber (5).

2.4. Virus titration:

Microtitration in cell culture microplates was carried out. 10-fold serial dilutions (10^{-1}-10^{-9}) were prepared and each dilution was inoculated into four wells. Appearance of the CPEs were carefully followed up on a daily basis for seven days. 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 MOI of 0.03, 0.03, and 0.05, respectively.

2.6. Evaluation of CPEs:

Inoculated cell monolayers were observed daily. Any progress in the quality and quantity of CPEs was recorded. MV-, RV-, and RSV-inoculated cell cultures were observed till day 7, 7, and 3 post-inoculation respectively, when the highest extent of CPE normally appears in the relevant control cell substrates. Samples for titration were also taken at the same time.

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2.7. The procedure for inoculation of viruses into control and test cell lines:

Inoculation of MV into Z1 and Vero-WHO: Z1 and Vero-WHO cell cultures were inoculated at the defined MOI. The formation of CPEs was carefully observed and recorded on a daily basis. The samples were taken from the supernatants when the inoculated cell cultures showed the maximum CPEs. Viral titers in the harvested materials were compared between two cell substrates. To check the reproducibility, the experiment was repeated three times.

Inoculation of RV into Z1 and RK-13: The same procedure, using Z1 and RK-13 cell lines.

Inoculation of RSV into Z1 and Hep-2: The same procedure, using Z1 and Hep-2 cell lines.

2.8. Interference test:

Inoculation of RV to permissive cell lines is not necessarily followed by the formation of distinct CPEs. The interference test is stablished to check the multiplication of RV in the absence of CPE. To do this, the RV-inoculated cell cultures are re-inoculated with VSV after 72-96 hours, if no CPEs have been seen. Observing CPE (cell lysis) after 24-48 hours means that the cell substrate is not permissive to RV, but the absence of cell lysis shows that the RV has successfully replicated in the cell substrate, even with no signs of CPE.

2.9. Data analysis:

CPE formation following inoculation of the viruses was qualitatively evaluated. Data on the viral titers in the harvested materials were analyzed using SAS 9.4, employing the probit procedure. Multiple comparisons of titers derived from different cell types were conducted using the Tukey test.

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3. Results

3.1. Inoculation of MV to Z1 and Vero-WHO cell lines:

CPEs, as the formation of syncytia, appeared 24 hours post-inoculation with a peak on day 7 post-inoculation (Fig 1). The mean of MV titer in the samples from inoculated Vero-WHO cells was 10 times higher than Z1-inoculated ones. The detailed data on the extent of CPE formation and the MV titer in harvested materials from three inoculation runs are summarized in Table 1. There was a significant difference ($P < 0.05$) between the MV titer in the harvested materials from the test and control cell substrates (Table 2, and Fig 2).

3.2. Inoculation of RV to Z1 and RK-13 cell lines:

CPEs following inoculation of RV are clamp-shaped views, but normally hard to see. They start to appear 48 hours post-inoculation with a peak on day 7 post-inoculation (Fig 3). The detailed data on the extent of CPE formation from three inoculation runs are summarized in Table 3. No CPEs were observed in Z1 cells even at day 7 post-inoculation, so an interference test using VSV was performed. The RV-inoculated RK-13 cells remained attached to the flask, but the RV-inoculated Z1 cells were completely detached from the flask, as the result of VSV replication (Fig 3). It shows the non-permissiveness of the Z1 against RV. The RV titer in the samples from RV-inoculated RK-13 and Z1 cells during three inoculation runs is summarized in Table 3. There was a significant difference ($P < 0.05$) between the RV titer in the harvested materials from the test and control cell substrates (Table 2).

3.3. Inoculation of RSV to Z1 and Hep-2 cell lines:

CPEs, as cell rounding, appeared 24 hours post-inoculation with a peak on day 3 post-inoculation (Fig 4). The detailed data on the extent of CPE formation and RSV titer in harvested materials

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during three inoculation runs are summarized in Table 4. There were no significant differences ($P < 0.05$) between the RSV titer in the harvested materials from the test and control cell substrates (Table 2, and Fig 5).

4. Discussion:

As obligate intracellular organisms, viruses could only be replicated in viable cells. Significant advances in virology have been made since cell culture was first used for the inoculation of viruses about one hundred years ago. Today, cell cultures are not only used in the isolation, identification, and propagation of viruses, but also they have a fundamental role in the diagnosis of several viral diseases and the production of many biological products including viral vaccines. In recent decades, different cell lines have been developed from a wide variety of species such as humans, animals, plants, etc. Evaluating the sensitivity of newly derived cell lines against different viruses and assessing their successful support of viral replication is among the primary studies on the developed cell lines. Permissiveness to a wide range of viruses, supporting viral replication and formation of distinct CPEs, is considered as distinguishing properties of a newly developed cell line.

Reptile virology, a relatively young field, has rapidly developed in the past decades. As the infection of reptiles with the members of different viral families including Adenoviridae, Herpesviridae, Paramyxoviridae, and Reoviridae has been well demonstrated (7), the permissiveness of the Lizard-derived cells against several human and animal viruses could be predictable.

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The Z1 cell line is successfully developed by RVSRI researchers. It is derived from the tail of the Cyrtopodian scabrum lizard. This cell is easily passaged and interestingly tolerates 30-45°C in the presence of 5% CO_2 in an incubator. The sensitivity of this cell line against some viruses was assessed, but the results have not been published in a peer-reviewed journal. This study aimed 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). No more data is available 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 used in MMR vaccine production for decades and their safety has been well documented. So, 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, there were enough skills and expertise on optimal cultivation conditions on selected viruses at RVSRI and TUMS. Thirdly, standard cell substrates as control cell lines (Vero-WHO, RK-13, and Hep-2) against under study cell line (Z1) were available. Moreover, characterized MV, RV and RSV were easily available at RVSRI and TUMS.

In this study, the optimal condition of virus cultivation, including MOI, was defined based on previous experiences and written procedures relevant to the selected cell lines and virus strains. A qualitative method (microscopic observation) and a semi-quantitative method (CCID50/ml) were used to evaluate the extent of CPE and viral titer, respectively. To ensure the validity of acquired data, all experiments were performed three times.

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It was revealed in the literature review that plenty of cell lines have been developed from different reptile species during the last decade, but no data on the evaluation of their permissiveness to viruses were found. On the other hand, no articles on the permissiveness of the Z1 and similar cell lines to viruses have been published yet. Accordingly, there was no chance to compare the results of this study with similar ones.

However, the acquired data of this research could be interpreted itself. The primary aim of this research was to evaluate the potential of the Z1 cell line for successful support of MV, RV, and RSV replication which may result in its application for virus isolation, diagnosis purposes, production of biological products, etc. Based on the results, the Z1 cell line is considered a suitable cell substrate for successful propagation of RSV. Despite a lower yield of RSV in comparison to Hep-2, the Z1 cell line still supports quick viral replication and forms distinct CPEs. More optimization runs may upgrade the yield and make it more applicable for RSV propagation. For the MV, the Z1 cell line supports its replication at sub-optimal conditions. Having very sensitive cell lines such as Vero cell lines particularly Vero-SLAM, using the Z1 cell line for MV-related research seems illogical. Z1 cell line does not support replication of RV at all. The exact receptor of RV is not defined yet, however, myelin oligodendrocyte glycoprotein (MOG), a member of the immunoglobulin superfamily, is introduced as a potential receptor of RV (9). It should be defined through molecular studies, if RV has no receptors on the Z1 or enters but cannot replicate. If non-permissiveness is due to a lack of receptor, then it could be interpreted that the Z1 cell line most probably does not support the other viruses that use the same receptor, as well.

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Authors' contributions

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

Our study does not involving any human or animal subjects/samples, so there is no ethical issues. However, ethical approval for performing the study was granted by the Ethics Committee of Razi Vaccine and Serum Research Institute.

Conflict of Interest

The authors declare no conflict of interest in this study.

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Data availability

All data generated or analyzed during this study are included in this article and its supplementary material files. Further enquiries can be directed to the corresponding author.

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

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

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

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

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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

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

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