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Original Article

Characterizing the BHK-21 C5 cell line and determining cellular sensitivity to rubella virus compared with the routine cell (RK13)

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Abstract

The World Health Organization has strict rules and recommendations on the selection and use of cell substrates in laboratories. Given the widespread use of safe and secure cell substrates in the production and quality control of viral vaccines and also the high demand for vaccines against viral diseases, obligating the selection of a suitable cell substrate for cultivation and production of biological products. Animal cell lines play a valuable role in the preparation and propagation of viral seeds; thus, the current study used the BHK-21 cell line among others for viral checking with the aim of replacing the BHK-21 C5 cell line with the RK13 cell line to investigate the cytopathic effects of the rubella virus. To this end, attempts were made to determine the characteristics of the BHK-21 C5 cell line including cell growth characteristics and sterility tests to validate its safety and security. Then, by culturing the cells in a 96-well microplate, titration of the rubella virus was subsequently performed by preparing serial dilutions of the virus from 10^{-1} to 10^{-5} and inoculated to cell lines in order to compare the sensitivity of BHK-21 C5 and RK13 cell lines to rubella virus. Data analysis according to the results of the tests by ahead default, *p*-value < 0/05 was equal to *p*-value = 0.01 based on SPSS analysis with the paired-sample t-test. In addition, the box-plot diagram indicated a significant difference between these cell lines. Based on the results, the BHK-21 C5 cell line seems to be more sensitive to the rubella virus than others. Therefore, it can be used for production and quality control of the vaccine and in research and diagnosis of rubella.

Keywords: characterization, BHK-21 C5 cell line, rubella virus, titration test, RK13 cell line

Caractériser la Lignée Cellulaire BHK-21 C5 et Déterminer la Sensibilité Cellulaire au Virus de la Rubéole par Rapport à la Cellule de Routine (RK13)

Résumé: L'Organisation mondiale de la santé a des règles et des recommandations strictes sur la sélection et l'utilisation de substrats cellulaires dans les laboratoires. L'utilisation généralisée de substrats cellulaires sûrs et sécurisés dans la production et le contrôle de la qualité des vaccins viraux ainsi que la forte demande de vaccins contre les maladies virales nous obligent à sélectionner un substrat cellulaire approprié pour la culture et la production de produits biologiques. Les lignées cellulaires animales jouent un rôle précieux dans la préparation et la propagation des graines virales; ainsi, la présente étude a utilisé la lignée cellulaire BHK-21 entre autres pour le contrôle viral dans le but de remplacer la lignée cellulaire BHK-21 C5 par la cellule RK13 pour étudier les effets cytopathiques du virus de la rubéole. À cette fin, des tentatives ont été faites pour déterminer les caractéristiques de la lignée cellulaire BHK-21 C5, y compris les caractéristiques de croissance cellulaire et les tests de stérilité pour valider sa sûreté et sa sécurité. Ensuite, en cultivant les cellules dans une microplaque à 96

puits, le titrage du virus de la rubéole a ensuite été effectué en préparant des dilutions en série du virus de 10^{-1} à 10^{-5} et inoculé à des lignées cellulaires afin de comparer la sensibilité de BHK-21 lignées cellulaires C5 et RK13 au virus de la rubéole. L'analyse des données selon les résultats des tests par défaut anticipé, la valeur p<0/05 était égale à la valeur p = 0.01 sur la base de l'analyse SPSS avec le test t pour échantillons appariés. De plus, le diagramme en boîte a indiqué une différence significative entre ces lignées cellulaires. D'après les résultats, la lignée cellulaire BHK-21 C5 semble être plus sensible au virus de la rubéole que les autres. Par conséquent, elle peut être utilisée pour la production et le contrôle qualité du vaccin et dans la recherche et le diagnostic de la rubéole.

Mots-clés: caractérisation, lignée cellulaire BHK-21 C5, virus de la rubéole, test de titrage, lignée cellulaire RK13

1. Introduction

Cell substrates have been selected for the production of biological products, especially vaccines, from decades before with the development of cell cultures. As the safety of biological products, including vaccines, is an interesting subject of concern to regulatory agencies, the cell-based substrate must pass through difficult legislation (1, 2). Thus, choosing the proper cell substrate and adapting the virus to that substrate depend upon the implementation process and safety considerations (1, 3). It should be noted that to respond to present and future needs in the face of emerging and recurrent viral diseases, it seems necessary to employ different cell substrates (4). Preparation of a thorough and detailed report for the characterization of cell substrates to be approved by quality control systems seems indispensable, because it would confirm the end product and acknowledge the absence of contamination. In addition, efforts to replace the optimized animal cell substrates to increase production capacity and reduce costs are increasing (5). According to the WHO guidelines, it is possible to use continuous cell lines in vaccine production (6). As we know, anchorage-dependent cells have various biotechnological utilizations, including their use in producing viruses for high-scale vaccination purposes (7). Moreover, cell culture provides the conditions for identifying the number of pathogens by virus isolation from cell culture (8). Different cell lines are used in propagation, and considering the effects of viruses based on their characteristics, the BHK cell line is one that can be obtained from ATCC and reference laboratories (9). Rubella virus (RV) replicates in a variety of cells, from a primary cell line to a continuous cell line (10, 11). The effects of viral contamination induced by rubella virus cultured on continuous mammalian cell cultures are cytopathic (10). Currently, RK_{13} cells are used as the cell-substrate of the rubella virus, which has no specific cytopathic effects on these cells (12). Therefore, in this study, the BHK-21 C5 cell line has been characterized as an animal cell line from the anchorage-dependent cell culture and investigated as a suitable substrate for examining its growth conditions and how rubella virus affects it.

2. Material and Methods

2.1. Cell Culture in the Flask

While observing sterile conditions, 1.5 ml of the BHK-21 cell seed and 1ml calf serum with 8 ml DEMEM media were added to a 25 cm^2 T-flask and incubated at 37 °C for 24 hours with 5% Co_2. Subsequently, experiments were performed to determine the cell characterization (13, 14).

2.2. Cell Characterization

For cell morphology, the cell monolayer which formed on the surface of the flask floor was examined morphologically, and the cell arrangement was investigated by inverted microscope (15). To determine doubling time, flasks were sampled at the same time interval (twice within 24 to 48 hours) and counting was done. Therefore, when cells were in a logarithmic

phase, the doubling time was calculated (16). To evaluate the homogeneity or uniformity of cell quality and quantity, first and last cryovial contents of the cell seed were evaluated (14). Cell seed was also evaluated for microbial, mycoplasma, and mycobacterial infections in specific media (thioglycolate and TSB, pplo broth, pplo agar, and Lowenstein) under different conditions (17, 18). For cell counting and viability, 50 microliters of diluted cell suspension, along with 50 microliters of trypan blue dye, were placed in a microtube and cells were counted using Neobar (19, 20).

2.3. Preparation of BHK-21 C5 Monolayer

The BHK-21 C5 cell seeds were transferred from the flask to a 96-well microplate by adding 10 ml DMEM media and calf serum and transferring the solution to a sterile tub before being gradually poured into each well of the microplate and then incubated. Investigation with invert microscopy confirmed the accuracy of cell monolayer formation in the wells (21).

2.4. Preparation of RK13 Monolayer

After the formation of RK13 confluent cell monolayer, cell seeds were prepared by trypsinization and culture media for culturing in the microplate according to the above-mentioned method for the BHK-21 C5 cell line (21).

2.5. Rubella Virus Preparation

The sample-tested virus was a batch of monovalent attenuated rubella virus prepared by the Viral Vaccines Department of Razi Institute. For reconstitution of the lyophilized form, 0.5 ml of cultured media (DMEM) was added to calf serum to eventuate a homogeneous form. Serial dilutions from 10^{-1} up to 10^{-5} of the desired virus were preparedby adding 900 µl cultured media. To achieve a uniform concentration of the stock sample, the rubella virus solution was shaken. In preparing the first dilution (10^{-1}), 100 µl of the stock of the intended virus was inoculated into the first one. The dilute coefficient in this experiment was 0.1 (22).

2.6. Susceptibility of BHK-21 C5 to Rubella Virus

To evaluate the susceptibility of BHK-21 C5 cells to the attenuated rubella virus inoculation, first, the cultured monolayers were investigated in terms of cell morphology and the absence of any contamination, and cell growth was examined microscopically. 100 µL of each prepared viral concentration from 10^{-1} up to 10^{-5} were inoculated into each well. Then the inoculated cell monolayers were incubated at 37 °C with 5% Co_2. After 24 hours, the emergence of the virus effect was examined microscopically and compared to the cell control (23). Thereupon the endpoint dilution of the virus that causes cytopathic effects in 50% of the cell culture media was estimated according to CCID50/ml and using the Spearman and Karber formula, with daily observations of cytopathic effects made over a 7-day period (24, 25). This procedure was repeated for another 10 experiments.

2.7. Susceptibility of RK13 to Rubella Virus

The method of inoculating the virus into the RK_{13} cell culture was similar to that used for the BHK-21 C5 cell culture (26), but because the rubella virus has had no specified cytopathic effects on RK_{13} cell cultures, the interference test was used to check for this difficulty. The vesicular stomatitis virus (VSV) with a dilution of 10^{-3} was inoculated into the wells. Given that rubella and VSV virus both have interference properties, the presence of the rubella virus prevented the cytopathic effects of the VSV virus. Therefore, wells without the cytopathic effects of VSV were considered positive in terms of rubella virus effects in RK_{13} cell culture (27).

2.8. Comparison of the Rubella Virus Titration in BHK-21 C5 and RK13 Cells

A comparative study of the sensitivity of both BHK-21 C5 and RK_{13} cell lines with the intended virus was performed using SPSS analysis. First, normality of the data distribution (titers of virus in each cell line during 10 separate experiments) was verified using a nonparametric test 1-sample Kolmogorov-Smirnov test and considering two assumptions: the first was the H_0 hypothesis based

on a normal data distribution, and the second was the H_1 hypothesis based on data distribution that wasn't normal. The proving of the H_0 hypothesis by obtaining a *p*-value > 0.05 was verified. Then statistical analysis paired-sample t-test was performed for 10 separate experiments on the titration of the rubella virus on each of the two cell lines. Consideration was given the H_0 hypothesis, based on there being no significant difference in susceptibility to rubella virus in these two cell lines, and the H1 hypothesis based on there being a significant difference between them. The proving of the H_1 hypothesis by a *p*-value < 0.05 was verified. The Box plot diagram was also used to show the significant differences between these two cell lines.

3. Results

3.1. Cell Characterization

A uniform and regular cell arrangement in the culture media of BHK-21 C5 cells was observed. Counting the cells at the regular time interval showed that cells were in the growth logarithmic phase. The doubling time and μ ratio were 20.5 h and 0.03, respectively. Sterility tests indicated that none of the bacterial, fungal, mycoplasma, or mycobacterial contaminations were in the cell culture. Cell viability was about 92%.

3.2. Viral Foci Formed in BHK-21 C5

First, morphological viral foci at each dilution from 10^{-1} up to 10^{-5} were formed at the cell monolayer surface in each well after 24 hours and were visible compared to the cell control (Figure 1).

Figure 1. Foci formed by virus on BHK-21 C5 cell line (dilution $10^{-4)}$ (left) and cell control (right) (10X)

3.3. Sensitivity of BHK Clone to Rubella

The presence of cytopathic effects of the virus on the BHK-21 C5 cell monolayer indicated its sensitivity as a more suitable substrate in different virus dilutions. As can be understood from Figure 1, these effects were clearly observed with cell death as well as the appearance of cell deformation by rounding and disruption of cell arrangement and intercellular spaces after 72 hours (Figure 2). In addition, the appearance of CPE dependent upon dilution of the dose of the virus was slower.

3.4. Rubella Virus Assay using BHK-21C5 and RK13 Cell Cultures

As shown in Table 1, the results of rubella virus titration by BHK-21C5 and RK13 cell cultures, viral titers were performed using BHK-21C5 cells in 10 trials, more than the RK13 cells.

3.5. Comparison of Rubella Virus Assay in BHK-21 C5 and RK13 Cells

Based on the comparison of these two cell lines using a nonparametric 1-sample Kolmogorov-Smirnov test, the distribution of data (virus titers) in the RK_{13} cell line was equal to 0.1 and in the BHK-21 C5 cell line was equal to 0.2. Therefore, the titers have a normal distribution. Moreover, using the paired-sample t-test, statistical analysis was done

based on the comparison of the significance of difference in virus titers in these cell lines. By obtaining a *p*-value = 0.01, the significant difference according to *p*-value < 0.05 was verified (Table 2). A significant difference was also represented by the Box plot diagram. As the diagram shows (Figure 3), the BHK-21C5 cell line was more susceptible to this virus than the RK13 cell line.

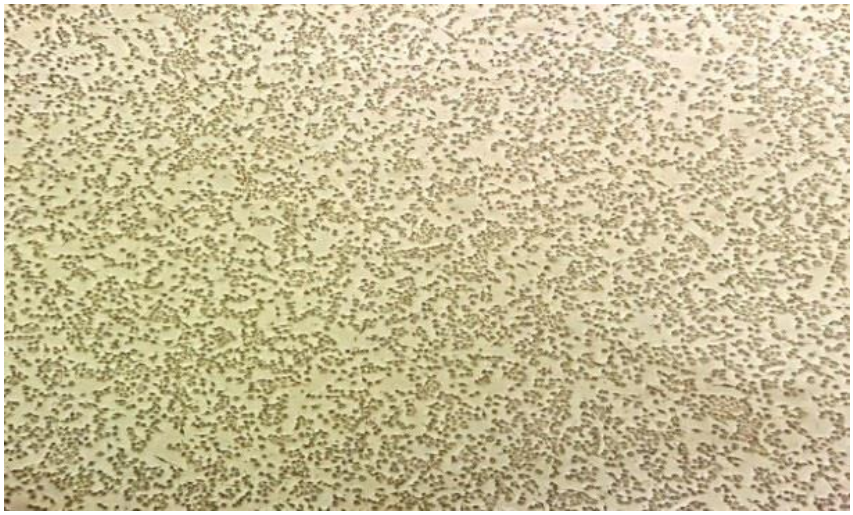

Figure 2. Cytopathic effects of rubella virus on BHK-21 C5 cell line by inverted microscope (40X)

Table 1: Different titers (CCID50/ml) of rubella virus on RK13 and BHK-21C5 cell lines

Titration frequency	Titration on RK13	Titration on BHK-21C5
1	$10^{3.75}$	$10^{3.62}$
2	$10^{3.37}$	$10^{3.62}$
3	$10^{3.62}$	$10^{3.37}$
4	$10^{3.25}$	$10^{3.75}$
5	$10^{3.50}$	$10^{3.81}$
6	$10^{3.37}$	$10^{4.00}$
7	$10^{3.25}$	$10^{3.62}$
8	$10^{3.37}$	$10^{3.75}$
9	$10^{3.62}$	$10^{3.87}$
10	$10^{3.25}$	$10^{3.62}$

Table 2: Comparison of mean titers of rubella virus on BHK-21C5 and RK13 cell lines

p-value	Standard deviation	Mean	Cell line
0.01	0.178	$10^{3.70}$	BHK-21C5
	0.173	$10^{3.43}$	RK13

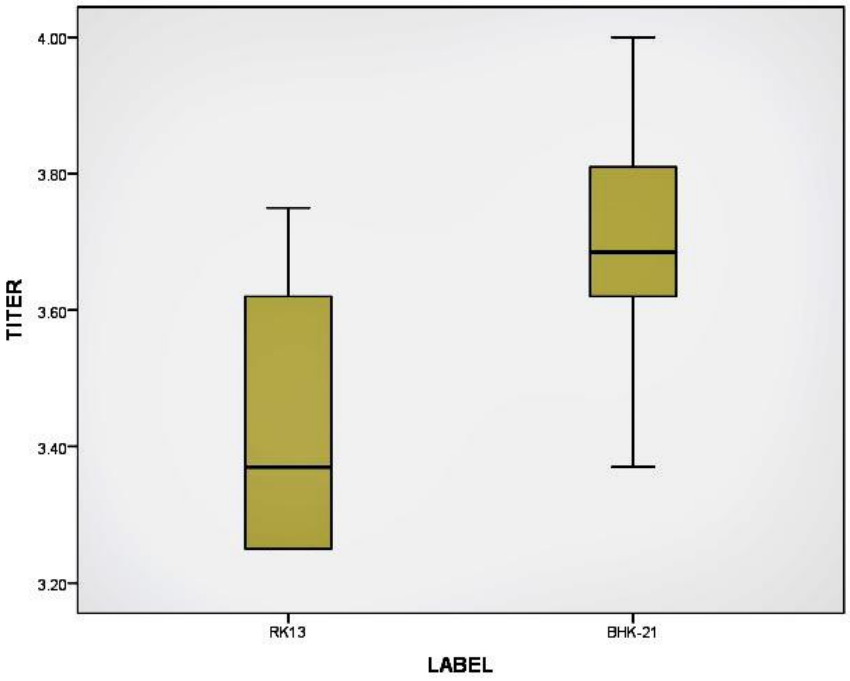

Figure 3: Comparative study of titration of BHK-21C5 and RK13 cell lines using SPSS analysis

4. Discussion

During the last fifty years, the choice of a suitable cell substrate for use in the production of biological products has been faced with much concern and attention. One main reason for these considerations is that the use of this particular substrate for humans must be safe (28). Appropriate features that these substrates should have include susceptibility to a wide range of viruses, the ability to proliferate an indefinite number of cells within a short period of time, and their features should be easily comparable (1). Furthermore, among the considerable advantages of cell cultures, the stability and reproducibility of the results obtained from them should be mentioned (29). Primary culture, diploid cells, continuous cell lines, and new cell substrates can be used as a form of monolayer or suspension in biological production (30). Continuous cell lines are immortal, and there are reasons for its widespread use (31). In addition, two critical factors important in optimizing the production of biological products from human and animal cell lines are the characterizing and testing of cell substrates to confirm the identity, purity, and suitability of cells (32). As is known from the past, hamster kidney continuous cell line has many applications in biotechnological products and veterinary vaccines. In this study, by choosing a clone of the BHK-21 cell line that serves as a cell substrate for many applications in veterinary vaccines and biological products, attempts were made to investigate the effects of the virus on said cell line (33). As the potential of infectivity of any viral product can be specified by virus titration, various dilutions of the intended virus were prepared and, after a specific period of time, the effects of the virus were observed (34, 35). Moreover, the viral infectious titers were measured during the final evaluation of the development of viral vaccine products according to the CCID50 unit (24). The microplate method was used in isolating a number of cultivable viruses, a simpler and more efficient method than traditional ones (36, 37).

Wang et al. studied the microtitration of rubella virus on rabbit kidney cell culture. Their results revealed the difficulties in identifying the cytopathic effects of the virus through microscopic tests (12). Some researchers have observed in their results that different strains of the rubella virus had various morphological plaques on the RK13 cell culture under the same conditions as the culture media (26). Previous studies of the cultures of different viruses on the BHK cell line have indicated its ability to be used as the culture of various viruses. Amadori et al. evaluated the phenotypic characteristics of BHK cells to produce a vaccine for foot and mouth disease. Their results showed that decreased levels of integration and the disappearance of actin fibers were affected by the stability of virus particles (38). Lalosevic et al. studied the rabies vaccine prepared with the BHK-21 cell line culture as a choice in the production of immunogenic vaccines for humans, and their results indicated its suitability (39). Sekar et al. conducted a study on the BTV virus. Evidence from the virus titers showed that the virus was widely compatible with the BHK-21 cell line and that it was established as a target cell line for the production of the BTV vaccine (40). Previous studies have shown that the BHK-21 cell line is suitable for the proliferation of some viruses. The aim of the current study was to replace the BHK-21 C5 cell line with the RK13 cell line to investigate the cytopathic effects of the rubella virus. Therefore, attempts were made to approve cell validation by culturing and characterizing the BHK-21 C5 cell line in terms of cell growth and morphology and sterility tests. The BHK-21 C5 and RK13 cell monolayers were cultured and investigated. Then, by preparing consecutive dilutions of the virus and inoculating it into the monolayers, the existence or absence of CPE effects on them was investigated. The number of infected wells with cytopathic effects was determined. Viral titers were calculated using the Kerber method. Then, a comparative study of the sensitivity of these two cell lines was done using SPSS analysis, the nonparametric 1-sample Kolmogorov-Smirnov test, and the paired-sample t-test. According to the quantity of p-value = 0.01, which was obtained based on assumption and was less than p-value < 0.05, the drawn Box-plot diagram shows that the BHK-21 cell line is more sensitive to viral titers than the RK13 cell line. Therefore, according to the results of statistical data and morphological observations of the effects of the virus on the cell lines, the cytopathic effects of the rubella virus were more obvious on the BHK-21 C5 cell line than on the RK13 cell line. As the defect on the RK13 cell line was detected using an interference test and VSV, the BHK-21 C5 cell line was used in this study to negate the need for an interference test to evaluate the cytopathic effects of the rubella virus. Thus, it seems reasonable to consider it as a suitable alternative substrate to the RK13 cell line for diagnosis, evaluation, and titration of the rubella virus.

Authors' Contribution

Study concept and design: S. S.
Acquisition of data: F. Z.
Analysis and interpretation of data: S. S.
Drafting of the manuscript: F. Z.
Critical revision of the manuscript for important intellectual content: S. S.
Statistical analysis: S. S.
Administrative, technical, and material support: S. S.

Conflict of Interest

The authors declare that they have no conflict of interest.

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