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Original Article

Development of Indirect Immunofluorescence Technique for the Identification of MRC5 Working Seed Cell

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ABSTRACT

Diploid and continuous cell lines are used to propagate viral vaccines. At Human Viral Vaccine Department of Razi Vaccine and Serum Research Institute, MRC5 diploid cell is used for the development of live attenuated measles, mumps, rubella, and three types of poliovirus vaccines. Additionally, three continuous cell lines (i.e., RK13, HeLa, and Vero) are applied in quality control tests. Accordingly, cell cross-contamination can occur at cell culture labs, hence controlling the identity and specificity of cells is essential. Indirect immunofluorescence is a sensitive, specific, and simple test for cell identification. The present study was designed to develop the in-house indirect immunofluorescence test (IIF) as follows: homemade polyclonal anti-MRC5 serum was prepared in rabbits, and cross-reactive antibodies to RK13, HeLa, and Vero cells were eliminated. The diploid and continuous cell lines were fixed on Teflon slide using cold methanol and acetone. The reproducibility of the in-house IIF test was evaluated using the agreement Kappa test. The purity of the three batches of MRC5 working seed cell at Human Viral Vaccine Department of Razi institute was verified using IIF and no contamination with continuous cell lines was detected.

Keywords: MRC5, Cross-contamination, IIF, Quality control test

Développement d'une technique d'immunofluorescence indirecte pour l'identification de la cellule de semence de MRC5

Résumé: Des lignées cellulaires diploïdes et continues sont utilisées dans la productiondes vaccins viraux. Au département des vaccins viraux humains du *Razi Vaccine et du Serum Research Institute*, la cellule diploïde MRC5 est utilisée pour la propagation de la rougeole atténuée vivante, des oreillons, de la rubéole et de trois types de vaccins antipoliomyélitiques. De plus, trois lignées cellulaires continues (RK13, Hela, vero) sont utilisées dans les tests de contrôle qualité. Parconséquent, la contamination croisée de cellule à cellule peut se produire dans les laboratoires de culture cellulaire et le contrôle de l'identité et la spécificité des cellules est essentiel. L'immunofluorescence indirecte représenteun test sensible, spécifique et simple pour l'identification des cellules. L'objectif de cetteétude était de développer untest d'immunofluorescence indirecte (IIF) interne. A cet effet, un sérum anti-MRC5 polyclonal a été produitchez le lapin et les anticorps réactifs aux cellules RK13,

Hela et Vero ont été éliminés. Les lignées cellulaires diploïdes et continues ont été fixées sur une lame de Téflon en utilisant du méthanol froid et de l'acétone. La reproductibilité du test IIF interne a été évaluée en utilisant le tst de concordance Kappa. La pureté des trois lots de cellules semencières MRC5, utilisés par ledépartement de vaccin viral humain de l'institut Razi, a été vérifiée en utilisant un test d'immunofluorescence indirecte et aucune contamination avec des lignées cellulaires continues n'a été détectée.
Mots-clés: MRC-5, Cross-contamination, IIF, Test de Contrôle de Qualité

INTRODUCTION

WI-38 and MRC5 (Jacobs et al., 1970) are non-tumorigenic diploid cell lines with a finite capacity for serial propagation, which are used to viral vaccines. Further, continuous cell lines are used in quality control testing labs. Although diploid and continuous cell lines are often propagated at different cell culture laboratories, some mistakes can cause contamination of continuous cell lines with diploid cells. Cell cross-contamination is a common mistake in cell culture laboratories and cell banks worldwide. Unfortunately, the incidence of misidentification and cross-contaminated cell culture is estimated up to 15-20% (Capes-Davis et al., 2010). The majority of cell cross-contaminations are associated with the first developed human cancer cell line, that is, HeLa (Masters, 2002; Buehring et al., 2004; Johnen et al., 2013). Therefore, it is necessary to routinely control the purity of diploid cell lines at cell culture laboratories. At Human Viral Department of Razi Institute, like other countries, MRC5 diploid cell is used for the propagation of live attenuated vaccines of measles, mumps (RS12 strain), rubella, and poliomyelitis. In addition, continuous cell lines including Vero, RK13 (Rabbit Kidney 13), and HeLa are employed for quality control tests. Despite the fact that MRC5 and the continuous cell lines are propagated at different labs, the continuous cell lines can contaminate MRC5 diploid cells. Several molecular and cellular tests such as isozyme typing, human leukocyte antigen (HLA) typing, karyotyping, and DNA fingerprinting are available for cell characterization, but indirect immunofluorescence test (IIF) is a sensitive, specific, and simple test. Therefore, an in-house IIF was developed for purity investigation and characterization of MRC5 cells. The in-house IIF requires a specific polyclonal anti-MRC5 antibody as the primary antibody and preparation of the fixed cells on Teflon slides.

MATERIALS AND METHODS

Cell preparation: MRC5 diploid cell (ATCC® CCL171™), RK13 (ATCC® CCL37™), HeLa (ATCC® CCL-2™), and Vero (ATCC® CCL-81™) were propagated according to the American Type Culture Collection (ATCC) protocols. Briefly, after washing in phosphate-buffered saline (PBS; 0.01 M, pH: 7.2-7.4), the cells were suspended in Dulbecco's Modified Eagle's medium (DMEM; Sigma-Aldrich) containing 10% fetal bovine serum (FCS; Gibco®), and the flasks were incubated at 37 °C.

Slide preparation: The desired appearance of the diploid and cell lines was inspected under an inverted light microscope (Freshney, 1993). The cell lines were separately suspended in PBS containing 2% carboxymethyl cellulouse, and 1.5×10^5 cells per well were placed on a 21-well Teflon slide (Biotech). The slides were allowed to dry at laboratory temperature. The cells were fixed with cold methanol and acetone (Sigma-Aldrich) for 10 min at –20 °C, respectively. The slide was stored at -20 °C until use.

Polyclonal rabbit anti-MRC5 serum: Five rabbits aged three months (weight: 1.5-2 kg) were intravenously immunized against whole MRC5 diploid cells (Table 1). Using a rubber policeman, the layer of the MRC5 cells was scrubbed to protect the complete shape of cell surface antigens. Blood samples were collected before immunization and every 10 days after

immunization. The titers of polyclonal anti-MRC5 sera were assayed by IIF test.

Indirect immunofluorescence test development: Goat anti-Rabbit IgG, including H and L chains conjugated with fluorescein isothiocyanate (FITC) (Abcam, ab6717), was utilized as the secondary antibody. Optimal working dilutions of polyclonal anti-MRC5 and FITC-conjugated Goat anti-Rabbit IgG were determined using a checkerboard. Two-fold serial dilutions (from 1:2 to 1:1024) of polyclonal anti-MRC5 serum and FITC-conjugated anti-rabbit (1:100, 1:200, and 1:300) in PBS were used. The wells containing fixed MRC5 or continuous cell lines were covered with 50 µL of each dilution of anti-MRC5 antibody. The slide was incubated for 60 min at room temperature in a moist chamber and then washed three times with PBS. The cells were covered with 50 µL per well of different dilutions of FITC Goat anti-Rabbit IgG and incubated for 30 min at room temperature in a dark humid place. The cells were washed three times with PBS and the brightness of the cells was inspected under immunofluorescence microscope through a 100x lens. The serum of an unimmunized rabbit was used as negative control and MRC5 cells without antibodies were used as autofluorescence control.

IIFT reproducibility: The intra-laboratory reproducibility of the in-house IIFT was examined on three batches of HeLa, Vero, RK13, and MRC5 cell lines. The tests were performed by optimal working dilutions of homemade rabbit polyclonal anti-MRC5 serum and FITC-conjugated Goat anti-Rabbit antibody (Table 2). The reproducibility of the in-house IIFT was evaluated using the agreement Kappa test. K coefficient (ranging from 0 to1) was considered as the index of agreement.

Characterization of working seed cell of MRC5: The test was performed on three randomly selected batches of MRC5 working seed cell (74-1, 78-1, and 71-4) using the in-house IIF test.

RESULTS

Polyclonal rabbit anti-MRC5 serum. The titer of polyclonal anti-MRC5 serum was measured using IIF test before, during, and after immunization, and the titers were negative, 1:64, and 1:512, respectively.

The optimal dilution of primary and secondary antibodies. Checkerboard was used to determine the optimal antibody dilution for the best results. The optimal dilutions of FITC-conjugated Goat anti-Rabbit and polyclonal anti-MRC5 were determined 1:200 and 1:512, respectively. Reproducibility of the in-house IIFT and cross-reactive antibody titers are presented in Table 2.

Characterization of MRC5 working seed cell. The results of IIF test on three batches of MRC5 working seed cell identified that the tested cells were MRC5 diploid cells and there were no cases of Vero, RK13, or HeLa contamination (Figure 1).

DISCUSSION

Misidentification and cell cross-contamination are two risks in all cell culture labs. Several methods have been used for the characterization of cell lines. Short tandem repeat profiling has become a standard for the intra-species identity testing of human cell lines (Barallon et al., 2010; Johnen et al., 2013). In addition, Smith in 2011 used matrix metalloproteinase-2 (MMP-2) to verify cross-reactivity of species, and Choong-Jae Kim developed a method to identify species by using whole-cell fluorescent in situ hybridization. Techniques that use polyclonal antibodies, especially indirect immunoassays such as IIF test, are sensitive tests because polyclonal antibodies, even those with low affinity, produce strong signals. In 1941 an immunofluorescence technique and assays based on immunofluorescence had been developed, consequently several multicolor fluorescence immunostaining techniques have been recently introduced (Brouns et al., 2002; Buchwalow et al., 2005; Suzuki et al., 2005; Frisch et al., 2011; Bloemberg and Quadrilatero, 2012). It is assumed that the accuracy of indirect immunofluorescence assay and other tests is often

similar, although additional tests may be needed for the detection of a specific pathogen. de Oliveira et al. (2013) compared flow cytometry and indirect immunofluorescence assay in patients with American tegumentary leishmaniasis. Comparative analysis of indirect immunofluorescence assay and flow cytometry test by receiver operating characteristic curve (ROC) showed that flow cytometry had comparable accuracy. Sensitivity and specificity of 86% and 77% were detected in flow cytometry test, respectively, while the IFA had a sensitivity of 78% and specificity of 85% (de Oliveira et al., 2013). Koosha et al. (2004) used

Table 1. Immunization schedule for the preparation of polyclonal rabbit anti-MRC5 serum

No.	Day	Function	Cell count per injection	**IIF titer**
1	0	Sampling	-	-
2	1st	IV injection	5×10^5	
3	6th	IV injection	5×10^5	
4	12th	sampling	-	1:64
		IV injection	1×10^6	
5	18th	IV injection	1×10^6	
6	24st	IV injection	1.5×10^6	
8	30th	Bleeding	-	1:512

IIF: indirect immunofluorescence
IV: intravenous

Table 2. Cross-reactivity of anti MRC5 serum dilutions against cell lines

		IIFT against anti- MRC5 serum dilutions										
Cell strain	**Batch/ Passage No.**	**½**	**1/4**	**1/8**	**1/16**	**1/32**	**1/64**	**1/128**	**1/256**	**1/512**	**1/1024**	**Cross-reactive Ab titer**
Hela	83-1/61	+	+	+	+	+	+	+	+	-	-	1/256
	83-2/64	+	+	+	+	+	+	+	+	-	-	1/256
	83-3/64	+	+	+	+	+	+	+	+	-	-	1/256
Vero	83-1/154	+	+	+	+	+	+	-	-	-	-	1/64
	83-2/154	+	+	+	+	+	+	-	-	-	-	1/64
	83-2/159	+	+	+	+	+	+	-	-	-	-	1/64
RK13	83-1/91	+	+	+	+	-	-	-	-	-	-	1/16
	83-2/91	+	+	+	+	-	-	-	-	-	-	1/16
	83-3/91	+	+	+	+	-	-	-	-	-	-	1/16
MRC5	71-4/17	+	+	+	+	+	+	+	+	+	-	1/512
	78-1/18	+	+	+	+	+	+	+	+	+	-	1/512
	74-1/18	+	+	+	+	+	+	+	+	+	-	1/512

enzyme-linked immunosorbent assay and indirect immunofluorescence assay in the diagnosis of human strongyloidiasis. The sensitivity, specificity, positive predictive value, and negative predictive value for ELISA were 93.5%, 96.1%, 72.9%, and 99.2%, respectively, and for indirect immunofluorescence assay, they were 87%, 90.1%, 49.4%, and 98.4%, respectively. Both assays showed false positivity in hydatidosis, ascariasis, and toxocariasis; nonetheless, this was less common with ELISA test, which using filariform larval antigen may be a sensitive and specific test for human strongyloidiasis and could be preferable to indirect immunofluorescence assay (Koosha et al., 2004). Reina et al. (2003) detected mumps virus in throat swab and urine samples from patients with mumps using indirect immunofluorescence assay and shell vial culture, and they reported that the two clinical samples were similar in efficacy.

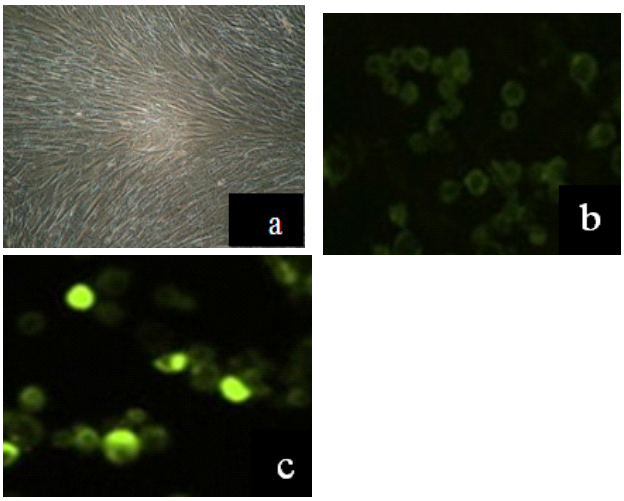

Figure 1. MRC5 identification by indirect immunofluorescence test (IIFT): a. normal MRC5 cells in a flask, b. processing of MRC5 cells before IIFT, c. processing of MRC5 cells after IIFT

Therefore, we developed an in-house IIF at Razi Institute that needs a homemade polyclonal anti-MRC5 antibody. Rabbits are common animals for specific immunization against antigens (Hanly et al., 1995). Accordingly, rabbits were immunized against MRC5 cells. Due to the granular nature of whole MRC5 cells, no adjutants were employed in immunization. The in-house IIF test was designed based on the cell lines (HeLa, Vero, and RK13) used in quality control tests. One of the factors affecting the quality of polyclonal antibodies is the phylogenetic distance between antigen and host (Stills, 2005). Since MRC5 and all continuous cell lines are derived from mammalians, it is recommended to prepare polyclonal anti-MRC5 serum in other animal species such as chicken.

The quick detection of cell cross-contamination plays a critical role in inhibiting the propagation of a wrong cell line because cell lines propagate faster than diploid cells. As a result, identification of master and working cell seeds of MRC5 is paramount and IIF test is a proper technique. In an IIF test, if all cells show adequate brightness, the purity of cells is identified and no more tests are required. Many tests are introduced for cell characterization, but the important factors for choosing a test are sensitivity, specificity, and rapidity.

Ethics

I hereby declare all ethical standards have been respected in preparation of the submitted article.

Conflict of Interest

The authors declare that they have no conflict of interest.

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