Original Article

An in-house indirect immunofluorescence test for ensuring that the cell bank is contamination free

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Abstract

Background: Cross-contamination between cells is a usual mistake at cell culture laboratories and cell banks worldwide. MRC5 diploid cell and Rk-13, Vero and Hela continuous cell lines are used in different stages of human viral vaccines propagation at Razi Vaccine and Serum Research Institute of Iran. However diploid and continuous cells are propagated at separated cell culture laboratory and continuous cells can contaminate MRC5 diploid cells. Therefore, a sensitive test is needed. World Health Organization recommends few molecular and cellular techniques to cell characterization. **Materials and Methods:** The present study was therefore designed to set up an indirect immunofluorescence (IIF) test as follows: Polyclonal anti-MRC5 cell and anti-rabbit antibodies were prepared in rabbit and goat, respectability. Anti-rabbit IgG was purified using protein G affinity chromatography, conjugated to fluorescein isothiocyanate (FITC), and then further purified to remove unbound FITC using Sephadex G 25 chromatography. Using double immunodiffusion assay, purification of homemade anti-rabbit IgG was asssayed by observation of a single arch. **Results:** The titer of homemade FITC conjugated goat anti rabbit IgG was measured 1/16 vs 1/8 of commercial type. Fluorescein/protein molar ratio of local made fluorescein goat anti-rabbit IgG was measured 3.44 and its protein concentration and FITC concentration were determined 1.198 mg/ml and 0.01 mg/ml, respectively. **Conclusion:** Moreover, homemade IIF test showed 100% intra-laboratory reproducibility. Purity of three batches of MRC5 working seed cell was verified using in- house IIF test and no contamination to continuous cell lines was found .

Keywords: MRC-5, cross-contamination, indirect immunofluorescence technique, Quality Control Test, human viral vaccine

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Introduction

There are two human diploid fibroblast cell strains, WI-38 and MRC5 which were widely used to propagate of viral vaccines, and many continuous cell lines have been derived from human and many different species of animals. MRC5 cell is a fibroblast like diploid cell that was developed from a 14-week male fetus lung tissue in 1970 and has been used for producing human viral vaccines (1). While, continuous

cell lines such as Vero, HeLa and Rk13 are used for quality control of viral products, however recently some continuous cell lines such as Vero have been permitted to be used for vaccine production (2). Therefore, both diploid and continuous cell lines are usually propagated at a shared cell culture laboratory that can cause cross-contamination of cells.

Cell to cell cross-contamination is a usual mistake at cell culture laboratories and cell banks worldwide. In a culture of diploid cells contaminated with continuous cell lines after few passages cell lines diploid cells completely displace because continuous cell lines grow faster than diploid cells and no evidence of diploid cells is found using an inspection of cell morphology under a light microscopy. Therefore, many of the cell to cell cross-contamination problems connected to continuous cell lines especially HeLa cell , as the first established human cancer cell line (4-6). First cell to cell cross-contamination of cells was reported in the 1950s (3). National testing services reported up to 36% of cell lines are incorrectly selected. In German cell bank, about 20% of reported cell lines were not true. Most cell to cell cross-contaminants often observe within the same species whereas interspecies contaminants include a small of cases (7). Therefore, it is necessary that cell culture laboratories routinely inspect purity of their cells. There are many tests for cell characterization such as karyotyping, isoenzyme analysis, and DNA barcoding (5,14), however goals of a laboratory limit diversity of cell types, therefore affecting the design of cell identification testing.

At cell culture laboratory of Razi Institute, MRC5 cell and three continuous cell lines (Vero, Hela and RK13) are used in different separated stages of vaccine production and quality control. MRC5 diploid cell is used to propagate of viral vaccines and continuous cell lines are used for quality control of vaccine. IIF method is a suitable test for purity investigation of MRC5 cell . Development of in-house IIF test needs an uncommon polyclonal anti-MRC5 antibody (primary antibody) and an expensive fluorescence conjugate antibody (secondary antibody).

Therefore, a local made IIF test was developed by using homemade primary and secondary antibodies.

Methods

Preparation of the Slide:

MRC5 (ATCC® CCL171™), RK13 (ATCC® CCL37™), HeLa (ATCC® CCL-2™) and Vero (ATCC® CCL-81™) cells were propagated according to ATCC protocols.

Suspensions of the cell lines (3×106 cell/ml) were separately prepared in PBS containing 2% carboxymethyl cellulouse. About 1.5×105 cells per well were placed on a cleaned 21-well Teflon slide (Biotech).The slides were allowed to dry at laboratory temperature for 20 minutes.

The cells were fixed with cold methanol and acetone (Sigma-Aldrich). The slide was air dried and stored at -20°C until use.

Antibodies: Homemade Primary Antibody

Polyclonal rabbit anti-MRC5 serum was prepared using a homemade schedule as follows: Five rabbits aged 3 months (1.5-2 Kg) were intravenously immunized against whole MRC5 cell. To reduce damage of surface antigens, the flask scrubbed with a policeman and MRC5 cells were dispensed by vigorously pipetting. Immunizations were performed weekly for a total of five iv injections, first two injections with 5×105 cells followed by three injections with 5×106 cell. Six days after fifth injection, a pool of sera was collected and titer of polyclonal anti-MRC5 antibody was assayed by indirect immunofluorescence technique using Sigma Aldrich goat anti-rabbit IgG-FITC.

Homemade Secondary Antibody: Preparation of Rabbit Gamma globulin

Gamma globulin of a rabbit pool sera (10 ml) were separated using ammonium sulfate salting out method (8, 9). First step proteins were precipitated using 820µl saturated ammonium sulfate (W/V), pH 7.2 (Sigma) per ml serum at 0°C,and then 45% saturated ammonium sulfate (W/V) per ml serum. The precipitated gamma globulin was resolved in 10 ml of PBS and was purified by dialysis overnight in PBS at 4◦C for 24h. The protein concentration was calculated spectrophotometrically with the solution absorbance at 280 nm and 260 nm using Christian Warburg equation:

Concentration (mg/ml)=(1.55-A280nm)-(0.77-A260nm)

The protein concentration was 17.5 mg/ml that was diluted with PBS to obtain a concentration of 10 mg/ml. The gamma globulin solution was sterilized by filtration through a 0.22µm syringe filter and kept at –20°C.

Preparation of Goat Anti-Rabbit IgG

A young male goat was immunized S.C with 10 mg/ml gamma globulin combined with complete Freund's adjuvant for first injection and incomplete Freund's adjuvant for others. Immunizations were performed every 2 weeks for a total of four injections, followed by three boosters every 3-4 days. Goat gamma globulin protein was separated using saturated ammonium sulfate salting out method (8, 9). After dialysis in PBS, anti-rabbit IgG was purified using Sephadex G-protein affinity chromatography (Amersham Pharmacia Biothech). Briefly, according kit's protocol, after preparation of HiTrap column and loading of sample, column was washed with elution buffer. Fractions were collected in a volume of 1ml per tube and the protein concentration was determined by absorbance at 280nm.

Titer of crude and ammonium sulfate treated polyclonal goat anti-rabbit antibody were determined by double immunodiffusion technique.

Preparation of Anti-Rabbit IgG Conjugate FITC Conjugate

The solution containing IgG was dialyzed in 0.1 M sodium carbonate-bicarbonate buffer at pH 9.0 (Sigma Aldrich) and protein concentration of anti-rabbit IgG has been measured using Christian Warburg equation (17 mg/ml). FITC was added to the protein solution (0.05 mg/ml) with gentle stirring for 24h at 4°C (10). Unbinding FITC was removed using Sephadex G 25 gel (Amersham Pharmacia Biothech) filtration.

Two bands were observed after washing the column with PBS because IgG conjugated FITC molecules were exited faster than of un-banded FITC. Fraction containing FITC conjugated IgG was collected and the absorbance was determined at 280 nm and 495 nm.

To increase protein concentration, homemade fluorescein IgG conjugate was mildly dehydrated using polyethylene glycol 6000 (Sigma Aldrich).

According to molecular weight of IgG (160KD), the IgG conjugated FITC solution was transferred to a membrane dialysis tube with 100 MWCO (molecular weight cut-off's) (11). The membrane dialysis tube was covered with polyethylene glycol 6000 for 2 hours at room temperature.

Antibody Titration

Double immune-diffusion assay was used to determine titer of homemade goat anti rabbit IgG FITC in PBS was prepared and placed in adjacent wells on 1% agar-agar (Merck) gel. Central well was filled with purified rabbit gamma globulin. The gel was incubated at room temperature in a humid container, and antigen and antibody were allowed to diffuse into the gel for 24- 48 hours (12).

To preserve of homemade secondary antibody, 1% (w/v) BSA (Sigma Aldrich) and 0.1% (w/v) sodium azide (Merck) were used. Homemade FITC conjugated IgG was dispensed into equal volume and kept at 2−8 °C in a dark place.

A commercial goat anti-rabbit IgG (whole molecule)-FITC (Sigma Aldrich, F9887) was used as positive control. Recommended titer was 1:160 according to manufacture instruction and the optimum working dilution was determined using IIF test.

IIF Test Development:

Titration assay was used to determine the optimum working dilutions of primary and secondary antibodies. Serial two fold dilutions of polyclonal anti MRC5 serum, homemade fluorescein goat anti-rabbit IgG and Sigma FITC goat anti-rabbit IgG were prepared in PBS at PH 7.2. We added 50 µL of anti MRC5 antibody dilutions per well containing fixed cells and slide was incubated for 60 min at room temperature in a moist chamber (19). After three times washing in PBS, 50 µL diluted homemade and Sigma FITC goat anti-rabbit IgG were placed in wells. After 30 min incubation at laboratory temperature and three times washing in PBS, brightness of cells was inspected under IF microscope through a 100x lens. The last dilutions of every antibody with bright fluorescence labeled cells, were taken as end point of titration. Serum of an unimmunized rabbit and unstained MRC5 cells were used as the negative and auto-fluorescence controls, respectability.

Reproducibility of the test:

The intra-laboratory reproducibility of homemade indirect immunofluorescence test was examined ten times using a homemade polyclonal rabbit anti-MRC5 serum as a positive sample and serum of an unimmunized rabbit as a negative sample. The tests were performed by optimum working dilutions of homemade fluorescein-conjugated goat anti-rabbit antibody.

Statistical analyses

The reproducibility of homemade indirect immunofluorescence test was evaluated using the agreement Kappa test. K coefficient (ranging from 0 to1) was used as index of agreement. All statistical analyses were performed using the SPSS version 20 software (SPSS, Chicago, USA).

Identification of working seed cells of MRC5

The test was performed on three randomly selected batches of MRC5 working seed cells (74-6, 78-6, and 72-2) of Razi Institute.

Results

Specificity Test of Homemade Antisera

The cross-reactive antibodies in polyclonal anti MRC5 serum that reacted to Vero,RK13 and Hela cell lines were eliminated by diluting in PBS. In 1/512 dilution of homemade polyclonal rabbit anti MRC5 serum, MRC5 cells were seen in a fully brightness, without a specific fluorescence with continuous cell lines.

Homemade Fluorescein Goat Anti-Rabbit IgG double immunodiffusion assay

Titer of crude goat anti-rabbit was 1/256 that reduced to 1/64 after treatment with ammonium sulfate, figure 1 shows the results.

The level of homemade fluorescein goat anti-rabbit IgG was measured 1/8 .Therefore, it was concentrated using a dialysis tube and polyethylene glycol 6000 and titer increased to1/16. The level of Sigma Aldrich fluorescein goat anti-rabbit IgG was determined 1/8~16 by double immune-diffusion test (Figure 2).

The purification of homemade fluorescein goat anti-rabbit IgG was identified by forming a single arch of precipitated antigen-antibody in the equilibrium region.

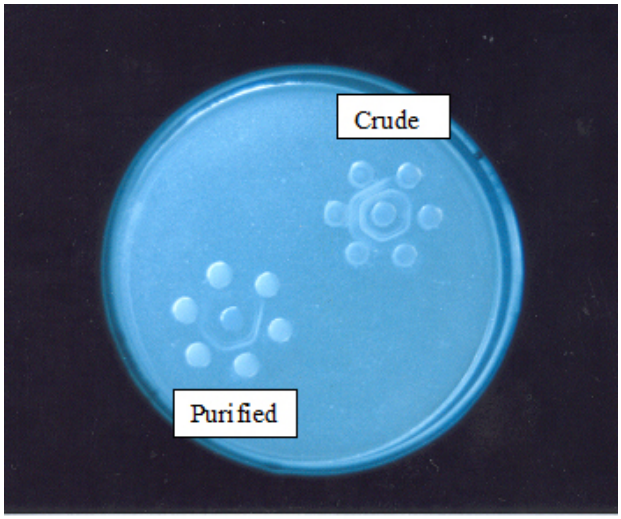

Fig. 1. The titer of crude and purified goat anti-rabbit antibodies by double immune-diffusion assay

FITC concentration

FITC concentration of a conjugate was determined at 495 nm and calculated from the following equation (10):

FITC concentration =A495/200 mg/ml

A495 of local made fluorescein goat anti-rabbit IgG was measured 2.01.Therefore, the FITC concentration was detected 0.01 mg/ml.

IgG concentration

A280 of IgG at a concentration of 1mg/ml is 1.4 and the protein concentration of homemade goat anti-rabbit IgG conjugate was calculated from the following equation (10):

IgG concentration (mg/ml) =[A280-(0.35×A495)]/1.4 mg/ml

Protein concentration of local made goat anti-rabbit IgG conjugate was determined 1.198 mg/ml.

Fluorescein/Protein Molar Ratio (F/P)

Proteins have an absorption maximum at 280nm. Fluorescein at 495nm has maximum absorption and at 525nm maximum emission. FITC covalently binds to free amino groups of proteins and unbound FITC was separated. Therefore, quality of FITC conjugate protein depends to Fluorescein/Protein (F/P) molar ratio that defined as the ratio of moles of FITC to moles of protein. Molecular weight of FITC is 390 Da and molecular weight of IgG is 160,000 Da.

F/P molar ratio was determined according to the follow equation (10):

(160,000/390) × (FITC concentration/ IgG

Fig. 2. IgG level of Sigma Aldrich and homemade fluorescein goat anti-rabbit by double immune-diffusion assay. Presentation of a single arch of precipitated antigen antibody in fluorescein goat anti-rabbit IgGs sample.

concentration)

A280 and A495 of homemade fluorescein goat anti-rabbit IgG were measured 2.38 and 2.0, respectively. Therefore , F/P ratio of homemade fluorescein goat anti-rabbit IgG was determined 3.44 that was in normal range between 2 and 4 (13).

Local made IIF Test Set Up

The optimum working dilutions of homemade and Sigma Aldrich fluorescein-conjugated goat anti-rabbit antibody were determined 1/300 and 1/240, respectively. The optimum working dilutions of homemade polyclonal anti-MRC5 was measured 1/512.

Intra-laboratory reproducibility

To evaluate the intra-laboratory reproducibility of homemade indirect immunofluorescence assay, positive and negative rabbit's sera were tested in ten independent runs. The calculated K coefficient was 1.00 that showed a 100% intra-laboratory agreement.

MRC5 Characterization

Results of IIF test on three batches of MRC5 working seed cell identified that tested cells were MRC5 diploid cell and there was no contaminations of MRC5 cell to RK13, Vero and Hela cells.

Discussion

Every cell culture laboratory is always at risk of entering a cell line to stock of other cell lines resulted in cell to cell cross-contamination. There are many cell identification techniques such as short tandem repeat (STR) profiling and DNA barcoding (5, 14) and every cell culture laboratory according to their facilities can select a technique. Therefore, we designed the present study to set up an indirect immunofluorescence test using local products of primary and secondary antibodies.

Three usual cell lines used to quality control tests of human viral propagation are HeLa,Vero,and RK13 cell lines.

Therefore, in-house IIF test was designed using RK13, Vero and Hela cell lines.

One of the factors in quality of polyclonal antibodies is based on phylogenetic distance between antigen and host. MRC5 and all continuous cell lines are derived from mammalian, therefore it was better to prepare polyclonal anti MRC5 serum in other animal species such as birds .

We used polyclonal antibodies because polyclonal antibodies even with low affinity are sensitive tools in immune responses assays (15). Direct and indirect methods of immunofluorescence

test have been introduced. Indirect method is time consuming than direct one , however due to binding of more than one labeled secondary antibodies to each primary antibody that strengthens the fluorescence signal, indirect method is more sensitive than direct method (16).

Most polyclonal antibodies are usually prepared in rabbits (17, 18) and in an indirect immunofluorescence test, surface molecules were labeled. Therefore, polyclonal anti MRC5 antibody was prepared in rabbits that immunized with whole scrubbed MRC5 cells. The cells were intravenously injected according to ATCC (American Tissue Cell Culture) protocol (19).

Commercial conjugate antibodies are expensive and routinely performance of an indirect immunofluorescence test needs to a stock of high quality of fluorescein conjugated antibody. Thus a fluorescein goat anti-rabbit IgG was prepared that its quality was as good as commercial conjugated antibody.

Fluorescein to antibody (F/P) ratio is a main factor that determines quality of a fluorescein labeled antibody. In fluorescence based tests, a sufficient level of flourchrome labeling allows high sensitivity in test. In over-labeling, fluorescent emissions from a flourchrome molecules are absorbed by other flourchrome molecules that are identified as flourchrome self-quenching effect. With a molar F/P > 6, an increased non-specific binding is usually observed that results in fluorescent background. In addition, over-labeling generally results in altered biological activity, specificity and solubility of the labeled protein (20).

Immuno-electrophoresis and double immune-diffusion assays are sensitive tests based on antigen-antibody interaction that are used to identity a specific antibody. In addition, double immune-diffusion is a simple technique for antibody titration. In present study, double immunodiffusion test was therefore used to specificity and purity evaluation of local made goat anti-rabbit IgG.

Coons et al. introduced immunofluorescence technique in 1941, however assays based on immunofluorescence have been developed and used at clinical and research laboratories (21, 22). Recently, several multicolor fluorescence immunostaining technique have been introduced (23-27) that are used to recognize more than one antigen in a sample.

The quick detection of a cell cross contamination plays a critical role in inhibition of propagation of a wrong cell line, because cell lines propagate faster than diploid cells. Therefore, identification of master and working cell seeds of MRC5 cells is important and IIF test is a proper technique.

Conflicts of Interest

The authors had no conflict of interest.

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