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Original Article

Use of Razi Bovine Kidney Cell Line for Proliferation of *Pneumocystis Carinii*

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ABSTRACT

Background and Objective: ***Pneumocystis*** **pneumonia (PCP) has been historically the most prevalent opportunistic infection in patients infected with the human immunodeficiency virus. Culture of the organism has not been faced with suitable success in artificial media, while various results have been reported for cell culture media. The aim of this study was proliferation of *Pneumocystis carinii* on the Razi Bovine Kidney (RBK) cell line and to compare growth rate with 'Vero' and 'MRC-5' cell lines.**

Materials and Methods: **We used 6 rats (Sprague-Dawley) provided from Razi Institute to infect with *Pneumocystis carinii* after suppressing the immune system with methylprednisolone acetate (40 mg/kg). Methylprednisolone acetate was used subcutaneously once a week for 8 weeks. Samples were homogenized after separation of the lung tissue. Microscopic examination was applied for prepared smears to confirm the presence of *Pneumocystis carinii*. Purified trophozoites were then inoculated into the cell line flasks. Growth rate was estimated by counting the trophozoite in each day.**

Results: **Number of cultivated organisms was increased after 5 days incubation in all applied cell lines. Growth rate of Vero, MRC-5 and RBK were 3, 3, and 3.75 times more respectively in comparison with number of the calculated cells in first day. Hence the difference between RBK and two other cell lines was significant (p = 0.023).**

Conclusion: **RBK cell line is suitable to proliferate *Pneumocystis carinii*.**

Key words: *Pneumocystis carinii*, Vero cell, MRC-5 cell, Razi bovine kidney cell

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Introduction

Pneumocystis is one of the important causes of morbidity and mortality in those patients with immuno-deficiency problems such as AIDS, malignancy, graft and also in those patients getting immunosuppressive drugs (1;2). Patients do not seem to have signs and symptoms after infection and early stages of organism replication (3). Pneumocystis has the highest frequency rate compared with other opportunistic infections in patients suffering from AIDS (4;5). At the present time, our information on the biology and pathogenicity of the organism is not suitable, although it is an important pathogen. Reported studies are limited due to the lack of suitable culture method and proliferation system for this organism (6). At the present time, there is not an agreement on the acceptable culture procedure to proliferate *Pneumocystis carinii* (7). *Pneumocystis carinii* is attached to type 1 alveolar epithelial cell in trophozoite form; hence its growth seems to be dependent on this type of cell. This kind of epithelial cells can not be used as continuous cell line since its primary form is very difficult to be obtained. Use of Vero cell line has been reported by Pifer (8). Bartlett has used WI-38 and MRC-5 (9). HEL-299 has been applied to culture of pneumocystis (10). Efficiency of the various host cells has not had satisfactory results for proliferation of the pneumocystis, based on reported studies on pulmonary and non-pulmonary cells.

Therefore, the aim of this study was to compare the efficiency of different cell lines for the best proliferation of the organism.

Materials and Methods

Preparation of rats for lung biopsy

Six female two months age rats (Sprague-Dawley) were prepared from Razi Vaccine and Serum Research Institute. The weight of all rats was 220-250 grams. These rats were kept for a week to ensure the lack of any bacterial contamination. They feed with UV treated food and sterile water containing 1 mg/ml of tetracycline and 0.25 mg/ml of ampicillin and 1.2 ug/ml of amphotericin B. Animal cages were continuously disinfected using alcohol and also autoclaved each day. Methylprednisolone acetate (Depo-Medrol, Pharmacia & Upjohn Co., Belgium) was injected subcutaneously once a week for 8 weeks (40 mg/kg) to stimulate pneumocyctis pneumonia in the lungs of test group (11). All rats were killed to obtain the specimens after eight weeks suppression of immune system.

Smear preparation

All lungs were separated from each rat under bio-safety cabinet after killing them and were put inside a sterile petri dish. The lung specimens were washed with PBS to remove access blood. Lung samples were pushed firmly on the slide to prepare suitable smears. All slides were stained with Giemsa method for microscopic examination of cysts and trophozoites (12).

Preparation of homogenized specimens

After removing fats and unwanted tissues, all remained lung tissues were sectioned. They were then mixed with 5 ml of PBS. The mixture was transferred into homogenizer. The homogenized cells were passed through sterilized gauze into the tube. Sodium citrate solution (10 mM, pH= 7.4) was added to the tube to adsorb calcium for prevention of cell clamping. Dithiothreitol (10 mM) was added to the homogenized buffer to reduce the viscosity of the mucus material of the brunch (13). Penicillin (200 U/ml), streptomycin (200 µg/ml) and amphotericin B (0.5 µg/ml) were added to the buffer to prevent contamination. Homogenized cells were centrifuged at 50 g for 5 min to separate those heavy lung slices. It was then centrifuged at 3600 g for 10 min at 10 ^{o}C and supernatant removed. Twenty milliliters of ammonium chloride (0.85%) was added to the mixture in order to solve the intra-cellular materials, lysis of blood cells and those cells containing nucleus. It was then incubated in bath water at 37 ^{o}C for 30 min. The mixture was then centrifuged at 3600 g for 10 min and the pellet was suspended in 10 ml of PBS. Finally the suspension was filtered with two different qualities respectively (5 and 10 µm of filter pore size). This step was repeated for second and third times to ensure the purity of homogenized cells.

Source of cell lines

In this research study, three cell lines were applied to culture *Pneumocystis carinii.* Vero and MRC-5 cells were kindly provided by Razi institute as follows: 1) The Vero cells were established from a normal African green monkey kidney and have

been continuously used for over 25 years to produce vaccines. Rapid growth and unlimited lifespan are two beneficial characteristics of most continuous cell lines. This cell line has been subcultured for 150-160 times for stabilization. It was then used for vaccine production in Razi institute, 2) The MRC-5 cell line was originally established from lung of human fetus. Morphology of the cell is fibroblast-like and normal diploid form. The cell was sub-cultured for about 25-30 times before being used for vaccine production in Razi institute, 3) RBK cell line has been registered in cell banks of Razi and Pasteur institutes with full name of "Iran-Razi-Khedmati" (IR.KH.BK). This cell line was primarily provided from Holoshtine race of male calf kidney. It was frequently proliferated for two years and incubated at 37 °C. Form of the RBK cell is polygon with circular nucleus looking poly-nucleus. It has been stabilized lasing for two months and sensitive to viral agents.

Counting method

For this purpose, 0.01 ml of specimens were put on clean microscopic slide and spread in 1 cm^2. Prepared smears were fixed with methanol after drying up and stained with Giemsa method. Trophozoites were counted by the Bartlet method (9) and its number was calculated in 1 ml by the following formula:

Number of trophozoite per ml = average number of trophozoite per microscopic oil field × number of microscopic fields in each cm^2 × dilution × 10^2

Number of the viable organisms was studied using Trypan blue staining method. One ml of homogenized specimens was put into each culture flask containing Vero, MRC-5 or RBK, incubated for 1 hour at 37°C. DMEM culture media supplemented with 10% PBS was added to flask and kept at 37 °C incubator. These flasks were checked daily under inverted microscope for any cell change and destroyed cells. Proliferation rates of *Pneumocystis carinii* were daily calculated after staining taken specimens from the flasks. For this purpose, 1 ml of culture media was taken and centrifuged at 3400 g for 10 min. Then, 0.01 ml of sediment was taken and stained with Geimsa. The tests were repeated several times to ensure reproducibility of the test.

Statistical analysis

Descriptive statistics and Kruskal-Wallis test were used to compare proliferation of trophozoites at fifth day for three cell lines. A p value less than 0.05 considered significant.

Results

Pneumocystis organisms were confirmed after microscopic observation of cysts and trophozoites in prepared smears from lung tissues (Figures 1-2).

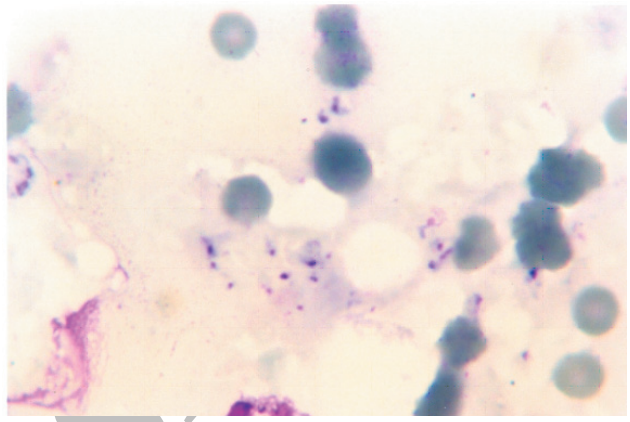

Fig. 1: Stained trophozoite by Giemsa method from lung smear

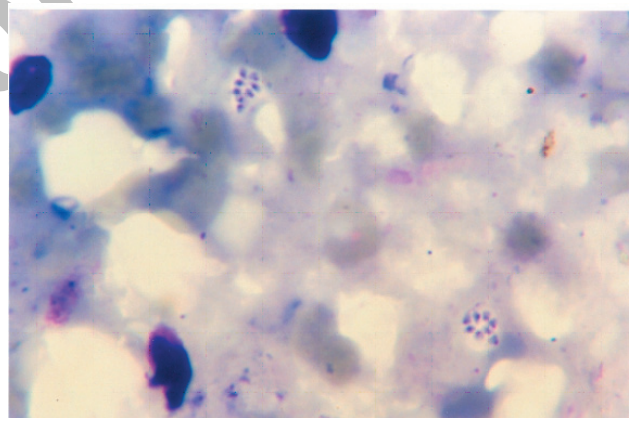

Fig. 2: Stained cysts by Giemsa method from lung smear

Vero cells

Number of the obtained trophozoites from rat lung was 4×10^5. Rate of viability of the organisms were approximately 2×10^5 (50%). Those *Pneumocystis carinii* cultured on Vero cell line showed 3 times increase during next five days in comparison with first day data (Fig. 3 and Table 1). Some vacant areas of the flask had progressive cell changes from day 3. Dying cells with accumulated nucleuses were observed around these areas. These areas were attached to each other with simultaneously increasing destroyed cells.

Table 1: Number of trophozoites and growth of *Pneumocystis* in three different applied cell lines at different days

Day	Viro			MRC-5			RBK		
	Trophozoite in supernatant	Increase in each day	Increase at time zero	Trophozoite in supernatant	Increase at each day	Increase at time zero	Trophozoite in supernatant	Increase at each day	Increase at time zero
0	2×10^5	-	-	2×10^5	-	-	2×10^5	-	-
1	2×10^5	-	-	2.2×10^5	1.1	1.1	3×10^5	1.5	1.5
2	2.5×10^5	1.25	1.25	2.5×10^5	1.25	1.13	3×10^5	1.5	-
3	3.5×10^5	1.75	1.4	3.5×10^5	1.75	1.4	4.5×10^5	2.25	1.5
4	4×10^5	2	1.14	4.5×10^5	2.25	1.28	5.5×10^5	2.75	1.22
5	6×10^5	3	1.5	6×10^5	3	1.23	7.5×10^5	3.75	1.36

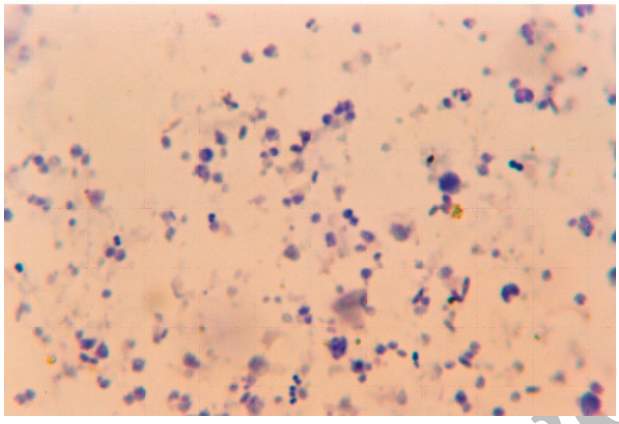

Fig. 3: Stained trophozoite by Giemsa in supernatant of cell culture

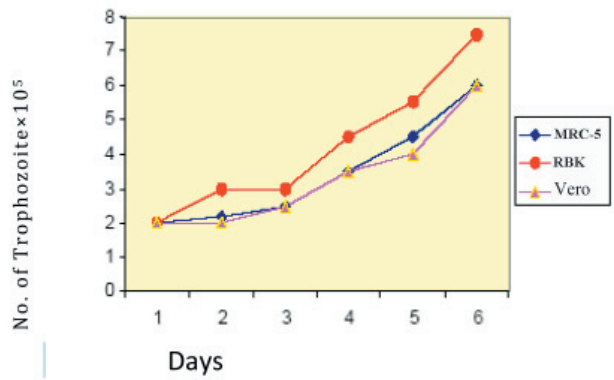

Fig. 4: Growth of *Pneumocystis carinii* in three applied cell lines; MRC-5, RBK and Vero.

MRC-5 cells

Those *Pneumocystis carinii* cultured on MRC-5 cell line showed 3 times increase during next five days in comparison with first day data (Table 1). Examination of flasks showed production of circular forms on monolayer of cell culture.

RBK cells

Cultured *Pneumocystis carinii* specimens were attached on cell line and produced large areas of destroyed cells of RBK. Number of trophozoites during next 5 days was 3.75 times greater in comparison with first day data (Table 1).

Comparing the collected results from cultures of *Pneumocystis carinii* (Fig. 4) revealed that its growth on RBK cell lines was higher than other two applied cell lines. Use of Kruskal-Wallis test confirmed that growth of trophozoites on day fifth was significantly higher as compared to growth of two other cell lines ($p = 0.023$).

Discussion

A large amount of live *Pneumocystis carinii* organisms free from any contamination is needed for any research on this organism. Studies on biological, biochemical, immunological and genetic aspects of *Pneumocystis carinii* need isolation of the organisms from lung tissue and purification of the organisms from any host cells. Having purified organisms are obviously necessary to study spread of the disease, pathogenesis, life cycle and treatment. In spite of all these necessities, there are not acceptable isolation and proliferation method for the organism from laboratory animal models or human clinical specimens. Current methods are far from reasonable standards (9).

At the present study, *Pneumocystis* organism was firstly isolated and cultured on cell line for next proliferations after purification. Looking after sterile conditions is quite important for collected specimens

from rat. Suppression of rat immune system causes invasion of all opportunistic organisms (bacterial, fungal and parasitic forms), resulting to death. *Pneumocystis carinii* is an opportunistic organism that proliferates in such situations. It is quite important to control the condition for prevention of disease in the studied rats from other opportunistic organisms. Since there are not enough facilities in our country to reproduce, none of the provided rats that were used in this study were not as specific pathogen-free (SPF). Therefore, it is difficult to set up a study with accurate results (13). We had not also enough facility to keep the animals in the room containing HEPA filter to have sterile space. Another problem that we faced was animal diet. It is recommended to use low protein diet (8%) to achieve suppression of immune system while we had to use ordinary ones (13), although it was reported ordinary protein diet could cause suitable situation to proliferate *Pneumocystis carinii* (10). All results showed less increase of cultured trophozoites on Vero and MRC-5 cells in comparison with other studies. Pifer cultured *Pneumocystis* on Vero cell and resulted to 10.8 times increase in its number in comparison with its culture at first day (8). Bartlett also used MRC-5 cell line and cultured *Pneumocystis* and reported 4 times increase in number of organism in comparison with its number at first day of culture (10), while our research proved its number increased only three times for both MRC-5 and Vero cell lines. In this research study, we used RBK cell line that is a native cell from our country. This cell line can be easily and rapidly proliferated and has also a recognizable form under microscopic field with a long life in culture medium. One of the other problems in this study was counting of trophozoites, because they attached to each other and produce clamping form that make counting difficult. Therefore, the classic counting method has not enough accuracy. In this research, we used microscopic counting method reported from Virginia Polytechnic institute that was used for anaerobic bacteria. We successfully tested RBK cell line in this study although our results were different for Vero and MRC-5 cell lines in comparison with other studies.

Although research on *Pneumocystis* can be designed from different aspects (14), but we believe that this research can be a good starting one for designing cell culture system, isolation and proliferation in our country. Lack of similar results on Vero and MRC-5 with other studies has been noticed by Cushion (12). On the basis of the Cushion report, *Pneumocystis* proliferation can not have the same result at different laboratories.

Conclusion

Quality of provided specimens from homogenated lung cells and dependency of trophozoites to it are the two parameters which affect culture and proliferation of *Pneumocystis carinii*. Cell systems should be selected to have the highest similarity to the host target cells in *in vivo* conditions.

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