MOLECULAR AND CYTOGENETIC CRITERIA FOR IDENTIFICATION OF SERUM-FREE CELL CULTURES

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ABSTRACT

The information about the authenticity of newly or already established animal cell cultures is essential. By using DNA analysis six cell lines has been investigated: 3T3, HeLa, McCoy, HEp-2, McCoy-Plovdiv and HEp-2-Plovdiv E. For the last four was made cytogenetic analysis confirmed that serum-free cell strains McCoy-Plovdiv and HEp-2-Plovdiv E are originally derived from McCoy and HEp-2 cell lines. PCR - analysis demonstrated that 3T3 cells are mouse originally, HeLa, HEp-2 and HEp-2-Plovdiv E are with the human origin and McCoy and McCoy-Plovdiv are hybrid cells carrying mouse and human genes in their genome.

Keywords: animal cell lines, serum-free cell cultures, McCoy-Plovdiv, HEp-2-Plovdiv E, cross-contamination

Introduction

Animal cell lines are important *in vitro* systems and tools for scientists in diverse biological disciplines as well serving the biotech industries. Currently, there are a large number of cell lines of human and other species that have inter - and intra-species cross-contamination (8, 10). The question of authenticity and characterization of cell lines is essential, especially after the shocking list of cell lines contaminated with HeLa cells (13), published in the 70's of last century. For years many cell lines that had not been well identified are exchanged between the laboratories worldwide. Two such cell lines are McCoy (16) and HEp-2 (11) isolated in the middle of 20th century. These cell lines are now in common use (4, 5), although it is known from many years of being contaminated with other cells. Several methods like cytogenetic, isoenzyme, DNA and immunophenotyping analysis has been developed for identification and characterization of cells (12, 15). Several efforts have been made to limit the spread and use of "unknown" cell lines. Different measures are implemented, including not accepting the publications in scientific journals, if it is not given accurate information about the authenticity of the used cells (9, 12). This has its basis, because it would save a lot of financial losses and emotional disappointments.

In the present study we used chromosomal and DNA analysis to verify the origin of two serum-free cell cultures McCoy-Plovdiv and HEp-2-Plovdiv E and to determine the species affiliation of six cell lines.

Materials and Methods

Cell cultures and growth conditions

Cell cultures 3T3 (mouse fibroblasts), HeLa (human adenocarcinom) and McCoy (mouse) were obtained from the National Bank for Industrial Microorganisms and Cell Cultures. Hep-2 (Human Epidermoid carcinoma, HeLa contaminant) was kindly provided by Dr. Trayancheva from the laboratory of virology, Plovdiv.

Cell lines 3T3, HeLa and Hep-2 were cultivated in DMEM medium supplemented with 10% FCS, while McCoy cells were grown in EMEM medium supplemented with 10%

FCS and antibiotics.

The cells were incubated at 37°C, 5% CO2 and high humidity in thermostat. The cultures were preserved in liquid nitrogen at -196°C previously transferred to sterile cryogenic tube in growth medium without antibiotics, enriched with 10% DMSO. For cell maintenance and experiments polystyrene cell culture flasks were used - 25, 75 cm^2 (Nunclon, Nunc).

Subculturing: Cells were grown for a long period of time by subcultures in every 5-6 days. A confluent cultures were trypsinized by 0.05% Trypsin 1:250 (Difco) and 0.02% EDTA (Sigma), dissolved in PBS. Cell number was detected in hemocytometer. The cell suspension was stained by 0.05% Trypan blue (Serva).

The serum-free cell cultures McCoy-Plovdiv and Hep-2-Plovdiv E were cultivated in DMEM/Ham's F-12 (1:1) medium. They were grown, subcultures and stored according to (4, 6).

Cytogenetic analysis

The preparations for chromosomal analysis were made on cells from: McCoy, Hep-2, McCoy-Plovdiv and Hep-2-Plovdiv E cell lines. Cells were cultured in plastic cell culture flasks ($75cm^2$). Colcemid 0.2 μg / ml was added at 48h. Following trypsinization cells were centrifuged, treated with 0.75% KCL for 10 min and fixed three times with methanol-glacial acetic acid (3:1) according to (7). The dried preparations were stained by 10% Giemsa solution. Chromosome preparations of metaphase spreading chromosomes has been selected and investigated, in order to define clearly their number and their morphology. In 50 metaphase plates was determined the total number of chromosomes and the number of the telocentric and "nontelotcentric" chromosomes (which include the metacentric and submetatsentric chromosomes) as is described by Defendi et al. (3). Preparations were examined with a Nikon Eclipse 80i microscope and documented by microphotography.

DNA-extraction

Genomic DNA has been isolated form the monolayer cell cultures of: 3T3, McCoy, Hep-2, McCoy-Plovdiv, Hep-2-Plovdiv-E, HeLa. Cell suspensions with $5x10^5$ cell/ml were centrifuged at 13000 rpm for 6 minutes. The cell pellets were resuspended in extraction buffer: (100 μl total volume of each sample) 10 μl PCR buffer 10x (100 mM Tris-HCl, pH 8,3; 500 mM KCl; 0.01 % gelatine), 10 μl $MgCl_2$ 25 mM; 4.5 μl 10 % Tween 20 in sterile distillated water; 0.6 μl proteinase K from 10 μg/ μl stock solution and 70.4 μl sterile distillated water. Samples were incubated at 60° C for 1 h, after that for 8 min at 95° C and stored at -20° C.

Primers

The following oligonucleotides were synthesized by LKB GmbH: primers N1 (GGGACGCTTGATGTTTTCTTTCC) and N2 (TCCTGAGACTTCCACACTGATGC), amplifying 130 bp from the human beta-globin gene and primers N3 (TTCCCCTGGCTATTCTGCTCAACC) and N4 (CGAACTCTTGTCAACACTCCACACACAG), amplifying 101 bp from mouse beta-globin gene.

PCR conditions

The PCR reaction include: 200 nM each dNTP; 0.6 μM N1 and N2 or 1.2 μM N3 and N4 oligonucleotide primers**;** 2 mM $MgCl_2$; 1.25 u Taq DNA polymerase (QIAGEN). PCR program include initial denaturation at 95°C for 8 minutes, followed by 27 cycles of denaturation at 95°C for 90 s, annealing at 65°C for 60 s, and final extension at 72°C for 10 min.

Electrophoresis

An aliquot of the PCR product was electrophoresed in a 1.75% agarose gel in 0.5 x TBE buffer (50 mM Tris, 50 mM Boric acid, 1 mM EDTA pH 8 in 1l of distillated water)

Results and Discussion

Figure 1A shows the distribution of the chromosomes in McCoy cells. In 50 metaphase plates were recorded chromosomes whose number varies from 52 to 74. In one cell was recorded polyploidy presented with 114 chromosomes. 29 of the cells have the same number of chromosomes - 65 (58% of all). These are cells with modal number of chromosomes. 84% of all tested cells have the number of chromosomes ranged from 63 to 67. Cells with different numbers of chromosomes are single. In serum-free cell culture McCoy the distribution of the chromosomes is similar, 50% of the cells were with modal number of 65 chromosomes (**Fig. 1B**). Two cells contained polyploidy chromosomes number. Also in the serum-free cell culture,

most of the cells (76% of all tested) were with the chromosomes in range of 63 to 67.

McCoy-cells:	1	1	1	1	4	2	29	5	2	1	1	1	1
Chromosomes:	52–55-58-	61-	63	64	65	66	67-	69-	71-	74-114			

1A

McCoy-Plovdiv-cells:	1	1	1	1	1	3	4	25	4	2	1	2	1	1	1	1
Chromosomes:	57	58	59-	61	62	63	64	65	66	67	68	69	70	71-	102-	128

1B

Fig. 1. Frequency distribution of chromosome content in 50 cells McCoy (1A) and McCoy-Plovdiv (1B)

Not only modal number of chromosomes, but also in the morphology of chromosomes was reported similarity between the two cell cultures (**Fig. 2**). The number of nontelocentric chromosomes varies in range of 14-18 per cell (**Fig. 2A, 2B**). It was also found the presence of dicentric chromosome with secondary constriction, giving proportions of 2:3:4. It is regarded as a chromosome marker characteristic of mouse cells McCoy B (3).

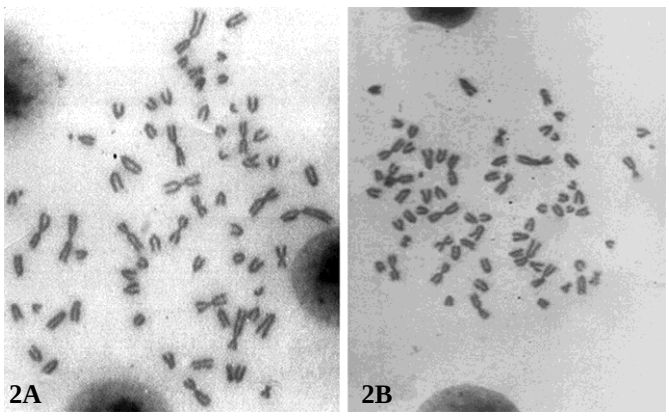

Fig. 2. Metaphase with modal number of 65 chromosomes from McCoy (2A) and McCoy-Plovdiv (2B)

The distribution of the chromosomes in HEp-2 and HEp-2-Plovdiv E cells are shown in **Fig. 3**. 75% of the investigated HEp-2 cells were with chromosomes in range 72-76.

HEp-2-cells:	1	1	3	1	3	1	3	8	12	10	5	1	1
Chromosomes:	60-62-68	69	70	71	72	73	74	75	76	77-131			

3A

HEp-2-Plovdiv E-cells:	1	1	2	2	2	3	3	11	2	5	3	4	2	3	4	2
Chromosomes:	54-58-62	63-65	66	67	68	69	70	71	72	73	74	75	76			

3B

Fig. 3. Frequency distribution of chromosome content in 50 cells Hep-2 (3A) and Hep-2-Plovdiv E (3B)

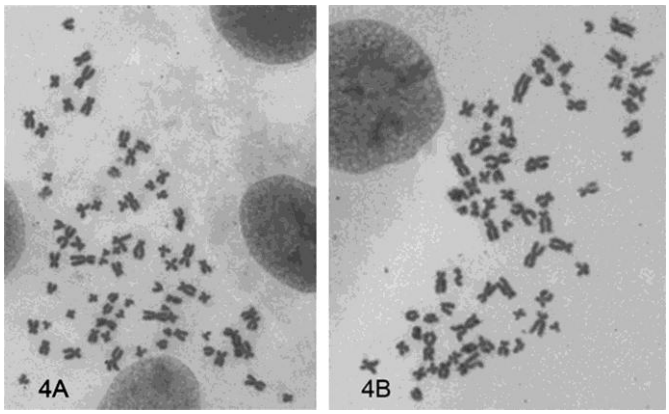

Fig. 4. Metaphase with modal number of 74 chromosomes from Hep-2 (4A) and 68 chromosomes from Hep-2-Plovdiv E (4B)

There was no clearly expressed peak of cells with modal number. 74 chromosomes were recorded in 12 cells (24% of all cells tested), 75 in 10 cells, 8 cells were with chromosome number 73. One of the cells is with a polyploid number of chromosomes. Three cells were of 68, three -70 and 3-72 chromosomes. In single cells were recorded 60, 62, 69, 71 and 77 chromosomes. **Figure 3B** is showing the distribution of the chromosomes of serum-free HEp-2-Plovdiv E cells, which is in the range 54-76 chromosomes. In single cells were measured at 54 and 58 chromosomes. In 11 cells was established chromosome number 68, which is 22% of all cells. Of 70 chromosomes were recorded in 5 cells. Other chromosome numbers were repeated at 2, 3 or 4 cells. In cell lines HEp-2 and HEp-2-Plovdiv E is predominantly amount of nontelocentric chromosomes (**Fig. 4**). Telocentric chromosomes present in the chromosome sets within 16-18.

The results obtained from the molecular analysis of the studied cell cultures are presented in **Fig. 5** and **Fig. 6**. In **Fig. 5** there are visible bands amplified from mouse beta-globin gene for cell lines McCoy, McCoy-Plovdiv and 3T3. There are no bands for HeLa, HEp-2 and HEp-2-Plovdiv E cell lines (**Fig.6**). Here, also is recorded the amplified product from genomic DNA of cells McCoy and McCoy-Plovdiv. No amplified product of genomic DNA from 3T3 cells in **Fig. 6**

The results of the molecular analysis confirm the origin of human cell lines HeLa, HEp -2 and HEp-2-Plovdiv E. Chromosome analysis verify the similarity between serum-free strain HEp-2-Plovdiv E and the originate cell line HEp -

2. Nontelocentric chromosomes dominated and telocentric are less than a quarter of all chromosomes in a cell. The differences in the distribution of chromosomes in 50 cells and the difference in modal numbers are probably due to adaptation of cells to the new conditions of cultivation.

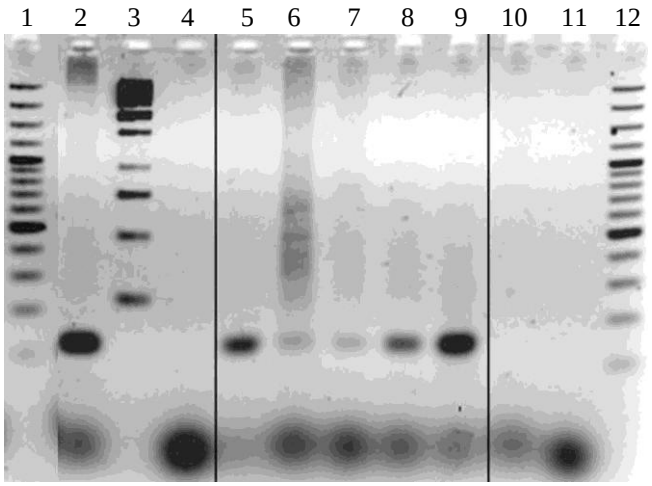

Fig. 5. PCR of the genomic DNA isolated from mammalian cell lines with primers N1/N2, amplifying 130 bp from the human beta-globin gene: **1**(100 bp DNA ladder), **2**(HEp-2), **3**(1 kb DNA ladder), **4** (N1/N2), **5**(Hep-2), **6**(McCoy), **7**(McCoy - Plovdiv), **8**(Hep-2-Plovdiv E), **9**(HeLa), **10**(3T3), **11**(N1/N2), **12**(100 bp DNA ladder)

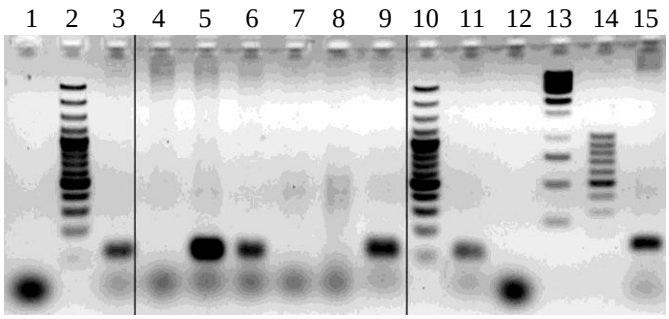

Fig. 6. PCR of the genomic DNA isolated from mammalian cell lines with primers N3/N4, amplifying 101 bp from mouse beta-globin gene: **1**(N3/N4), **2**(100 bp DNA ladder), **3**(3T3), **4**(Hep-2), **5**(McCoy), **6**(McCoy - Plovdiv), **7**(Hep-2-Plovdiv E), **8**(HeLa), **9**(3T3), **10**(100 bp DNA ladder), **11**(3T3), **12**(N3/N4), **13**(1 kb DNA ladder), **14**(100 bp DNA ladder), **15**(HEp-2 +N1/N2)

In the literature there is evidence that the cell line HEp-2 was contaminated with HeLa cells (2, 13). In this study we are not presenting an accomplished analysis in order to demonstrate the HeLa marker chromosomes and specific isozymes (13), witch can confirm that in this serum-free strain the cells are bearing these characteristics. While this is not confirmed, we presume that cell line HEp-2-Plovdiv E is also HeLa-contaminated. The results of the caryologic study present that serum-free cell line McCoy-Plovdiv originally derive from McCoy cell line. They have the same karyotype, in which is dominated the telocentric chromosomes. Modal chromosome number 65 is the same for both cell lines. Our results corresponded with those published by (3) relating to cell McCoy B (mouse) (1). The PCR analysis also confirms this result. While the 3T3 cells are proved as mouse cells, for the McCoy and McCoy-Plovdiv cells is obviously the presence of genetic material containing human genes. This has been confirmed by the PCR amplification with primers for human globin gene. In the literature there is evidence that McCoy cells are expressing human CD4 receptors (14). This was confirmed for McCoy-Plovdiv cells (unpublished results). McCoy cells were isolated in 1955 (16) "from the synovial fluid in the knee joint of a patient suffering from degenerative arthritis ". Five years later has been shown that cells distributed between laboratories worldwide are contaminated with mouse cells (3). When and under what circumstances became the mixing of human with mouse cells? Probably this will never understand, as commented in (13) because it is the result of targeted laboratory experiment, and mostly unconscious human error in the process of cultivation of different cell cultures simultaneously. Masters found that when it is mixing two cell cultures by the time it establishes a sustainable, which becomes dominant and displace the other and there are no evidence of somatic cell hybridization in cell cultures contaminated with other cells (9). In the case of McCoy cells suggest that faster growing cells are mouse L-cells (3). We can assume that they have stumbled across cultures of human synovial cells and McCoy shifted them as more aggressive and faster growing under the same conditions. But the results show that unless the process of displacement of cells there is a process of mixing of genetic material because McCoy cells contain except mouse and human genetic material into their genome. One likely assumption would be that between these two types of cells had a moment of interaction of their genetic material. The resulting hybrid has undergone rearrangement of the genetic material to obtain one in which there are expressed genes of the genomes, mouse and human. Due to the lack of correct information related to the process of contamination of these

cells, reasoning certainly has speculative nature. But we received evidence argument in support of the view that McCoy cells are hybrid cell line as indicated (14). This also applies to the serum-free strain McCoy-Plovdiv, cell line derived from McCoy. Still many cell lines are published without indicating that they are contaminated with other cells, although it is known for a long (10, 13). Our vision is in line with the proposal of the Masters (9, 12), that information about the authenticity must be correctly reported in published studies involving cell cultures. In this context we think is correct McCoy cells and McCoy-Plovdiv to be reported as "hybrid" cell lines, not as a "mouse fibroblasts" or "human synovial cells", as is usually present in the McCoy cell line publications.

Conclusions

a) Serum-free cell strains McCoy-Plovdiv and HEp-2-Plovdiv E were obtained respectively from the McCoy and HEp-2 cell lines;

b) Cell lines: HeLa, HEp-2 and HEp-2-Plovdiv E are human in origin;

c) Cell lines: McCoy and McCoy-Plovdiv are hybrid (contain mouse and human genes);

d) Cell line 3T3 is mouse originally.

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REFERENCES

1. **ATCC** (American Type Culture Collection) (1985) Catalogue of cell lines & Hybridomas. **5 edt**. Rockville.
2. **Chen T. R.** (1988) Genetics, **48**, 19-24.
3. **Defendi V., Billingham R. E., Silvers W.K and Moorhead P.** (1960) J. Nat. Cancer Inst, **2**, 359-385.
4. **Draganov M., Fransazov St., Draganov D., Murdjeva M., Popov N.** (2008-2009) J Culture Collections, **6**, 112-121.
5. **Draganov M., Murdjeva M., Michailova-Topalska T.** (2004-2005) J Culture Collections, **4**, 3-16.
6. **Draganov M., Murdjeva M., Sarafjan V.** (2003) Cytotechnogy, **42** (2), 163-167.
7. **Hozier J. C., Sawyer R and Moore M. M.** (1989) Mut. Res., **214**, 195-9.
8. **MacLeod R.A.F., Dirks W. G., Matsuo Y., Kaufmann M., Milch H., Drexler H. G.** (1999) Int. J. Cancer, **83**, 555–563.
9. **Masters J.** (2002) Carcinogenesis, **23**, No. 2, 371.
10. **Masters J.R.W.** (2000) Nature Rev. Mol. Cell Biol., **1**, 233–236.
11. **Moore A.E., Sabachewsky L. and Toolan H.W.** (1955) Cancer Res., **15**, 598 - 602.
12. **Nardone R.M.** (2007) Published online by Cell Biology and Toxicology, free toview @ 10.1007/s10565-007-9019-9.
13. **Nelson-Rees W. A., Daniels D. W. & Flandermeyer R. R.** (1981) Science, **212**, 446-452.
14. **Nogueira Y. L., Oliveira C. A. F., Ferreira A. G. P., Nakamura P. M., Magnanelli A. C.** (2003). Rev. Inst. Med. Trop. S. Paolo, **45** (4), 205 - 211.
15. **O'Brien S. J.** (2001) Proc Natl Acad Sci USA, **98**, 7656-7658.
16. **Pomerat C. M., Kent S. P., Locie L. C.** (1957) Z. Zellforsch., **47** (S), 158 - 174.