

Research Article

ISOENZYME ELECTROPHORESIS AND KARYOTYPE TECHNIQUES AS AN ALTERNATIVE FOR CELL LINE IDENTITY CONFIRMATION

Jéssica Z. Machado, Ana C. S. de Miranda, Claudia R. Gonçalves, Tamiko I. Ikeda, Aurea S. Cruz*

Núcleo de Cultura de Células, Centro de Procedimentos Interdisciplinares, Instituto Adolfo Lutz, Adress: Av. Dr. Arnaldo, 355, prédio da Virologia - 2º andar Cerqueira César - 01246-902 - São Paulo, SP - Brasil. Phone: (11) 3068-2910.

ABSTRACT

The use of cell culture in biomedical science has been constantly enhanced and the use of certified and microorganisms-free cell lines is indispensable. Due to the value and the difficulty of acquiring a commercially available kit (Authentikit™ system) for our routine in addition with the purpose of monitoring and ensuring quality of provided cell lines, and also contributing with cell characterization, the Núcleo de Cultura de Células do Instituto Adolfo Lutz (NCC- AL) padronized two techniques to identify the animal species from which the cells were originated: isoenzyme electrophoresis and karyotype. Cell extracts from 14 different cell lines were run under horizontal electrophoresis in agarose gel and lactate dehydrogenase isoenzyme profile was revealed using tetrazolium salt. The number of bands and the migration distances between bands of cell lines were similar to the literature, but small adjustments were needed to assist in the visualization of the bands that were not visible. Cell flasks for karyotype were incubated with colcemid solution and modal values of each cell line were calculated. Comparing all the results obtained with those reported in the literature, we conclude that both methods are promising and will allow better control of the services provided by the laboratory, not to mention guaranteeing authenticated cell lines for research and diagnostics.

Keywords: cell line, isoenzymes, karyotype, lactate dehydrogenase, cellular characterization.

Received in February 26, 2014 - Accepted in April 15, 2014 - Published ahead of print in April 17, 2014

INTRODUCTION

Cell culture is widely used in biomedical science such as vaccine production, cell biology, carcinogenic cell studies, study of chemical compounds and drugs effects in specific cells, biological products synthesis, among others (Rojas et al. 2008; Capes-Davis et al. 2010; Dittmar et al. 2010).

Whichever are the means for cell use, it is essential to verify their identity and if they have been cross contaminated. Since 1960, cross contaminated or misidentified cells have been used repeatedly, representing a frequent problem in culture cells and compromising studies (Markovic & Markovic 1998; Nardone 2007; Losi et al. 2008).

Several methodologies can be used to confirm authenticity of cell lines, for instance: isoenzyme electrophoresis, karyotype, fluorescent antibody staining and several DNA techniques (ATCC, 1992; Steube et al. 1995; Milanesi et al. 2003).

Isoenzyme electrophoresis is an efficient and fast

technique consisted of isoelectrical separation of specific intracellular protein families whose migration pattern allow viewers to distinguish the difference between the species. Isoenzymes are different molecular forms from enzymes, that display the same specificity for the determined substrate (ATCC 1992; Stacey et al. 1992; Steube et al. 1995). This method is recommended to verify interspecific cross contamination when at least 10% of total cells are contaminated (Fernandes & Simoni 1995; Nims & Herbstritt 2005).

Many cell banks use Authentikit™ system (Innovative Chemistry, Marshfield, MA, USA), an isoenzyme electrophoresis commercial kit (Stacey et al. 1997; Nims et al. 1998; Cabrera et al. 2006) however, for our laboratory the kit is unattainable, and its use is a regular basis.

Karyotype is a method used to identify specific chromosome markers, the genus and species of the animal, to analyze the characteristic chromosome number of each species and also determine common aneuploid in cell lines (Markovic & Markovic 1998; Wenger et al. 2005, Ramya 2009; Dittmar et al. 2010; Leandro & Cruz

* Corresponding author.

E-mail address: aucruz@ial.sp.gov.br

2012). It's a method that takes more time and depends on an expert ability. It's sensitive and provides valuable data in cell identity confirmation (Li et al. 2009; Dittmar et al. 2010).

In order to control the quality of cell lines maintained on laboratory stock and to guarantee its authenticity, this work purpose was to systematize karyotype to observe occurrence of analyzed cell lines chromosome number variation and standardize isoenzyme analysis to compare the migration pattern and number of bands with the results achieved by other cell banks with the use of commercial kit.

MATERIALS AND METHODS

Cell lines: 14 cell lines from different animal species were tested for lactate dehydrogenase (LDH) electrophoresis and karyotype (Table 1). Cell lines were kept at 37 °C on an appropriate medium, serum and without antibiotics (Freshney 1994; Miranda et al. 2011).

Table 1: Cell lines used, tissues or organs of origin, animal species and culture medium.

Cell Line	Origin	Species	Culture Medium
HeLa	Ephitheloid carcinoma of cervix	Human	MEM ^a with 0,1 mM AANE ^b + 1 mM P ^c and 10% FBS ^d
Caco-2	Adenocarcinoma of colon		Medium 199 with 0,1 mM AANE ^b and 15% FBS ^d
NCI-H292	Pulmonary mucoepidermoid carcinoma		Medium RPMI 1640 with 10% FBS ^d
RD	Rhabdomyosarcoma embrionary		MEM ^a + L-15 with 15% FBS ^d
MRC-5	Fetus lung		MEM ^a + L-15 with 15% FBS ^d
LLC-MK2	Kidney	Rhesus monkey	MEM ^a with 0,1 mM AANE ^b + 1 mM P ^c and 10% FBS ^d
Vero	Kidney	African green monkey	Medium 199 with 0,1 mM AANE ^b and 10% FBS ^d
NCTC clone 929	Connective tissue	Mouse	MEM ^a with 0,1 mM AANE ^b + 1 mM P ^c and 10% FBS ^d
SIRC	Corneal	Rabbit	MEM ^a + L-15 with 15% FBS ^d
PK(15)	Kidney	Porcine	MEM ^a with 0,1 mM AANE ^b + 1 mM P ^c and 10% FBS ^d
E.Derm	Derm	Equine	MEM ^a with 0,1 mM AANE ^b + 1 mM P ^c and 15% FBS ^d
MDCK	Kidney	Canine	MEM ^a with 0,1 mM AANE ^b + 1 mM P ^c and 10% FBS ^d
MDBK	Kidney	Bovine	MEM ^a with 0,1 mM AANE ^b + 1 mM P ^c and 10% FBS ^d
BHK-21	Kidney	Syrian hamster	MEM ^a with 0,1 mM AANE ^b + 1 mM P ^c and 10% FBS ^d

^a Minimum essential medium, ^b Non essential aminoacid, ^c Sodium piruvate, ^d Fetal bovine serum.

Cell extracts: extracts were prepared based on the *Laboratory Procedures for Animal and Human Cell Lines'* protocol. Cell line flasks with confluent monolayers were harvested with tripsyn and resuspended in appropriate medium. Obtained cell suspension was centrifuged (150 x g for 5 minutes) under refrigeration and cell pellets were washed with PBS (Phosphate Buffered Saline) solution at 4 °C, repeating this process twice.

Pellets (1-6x10⁷) were resuspended in 200 µl of extraction buffer (2% Triton X-100 in 50 mM Tris and 1mM EDTA, pH 7.5). Suspension was maintained on ice during 15-20 minutes. Lysate cells were centrifuged at 1900 x g during 5 minutes at 8 °C and supernatant was stored at -70 °C.

Isoenzyme electrophoresis:

LDH electrophoresis was made in horizontal LCV 7x8 apparatus (Loccus Biotecnologia, Cotia, SP, Brasil) using 2% agarose gel (Ultrapure™ Agarose, Invitrogen, Carlsbad, CA, USA) in 0.375 M Tris buffer (Invitrogen) pH 8.8 and 25 mM Tris/0.19 M glycine (Invitrogen), pH 8.3 as electrophoresis buffer. Cell extracts were applied on gel with 0.06% bromophenol blue / 10% glycerol on 3:1 proportion and ran for 2 hours at 100 V and 4 °C. Bands were revealed using the appropriate LDH stain solution (0.05 M Tris pH 7.5; 0.1 M Sodium lactate; 1.5 mM β-NAD (β-nicotinamide adenine dinucleotide); 0.24 mM MTT (3-(4'-5'-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide); 0.13 mM PMS (Phenazine methosulfate) (Sigma Chem. Co., St. Louis, MO, USA).

During band staining, gels were kept in a dark incubator at 37 °C for 20-30 minutes. Distance between bands was measured from the middle of origin point to the middle of the band. NCTC clone 929 and HeLa cell lines were used as standard and control, respectively (ATCC 1992; Miranda et al. 2011).

Karyotype analysis:

Cell lines flasks in exponential phase (at least 24h after sub cultivation) were incubated with 0.08 µg/mL colcemid (Merck S.A., Darmstadt, Germany) for 4-6 hours at 37 °C. Cells were resuspended in appropriate medium and centrifuged at 1000 r.p.m. for 5 minutes. A volume of 1mL of hypotonic solution (0.075 M KCL, Merck S.A) was added to the pellet each 3 minutes, until 4 mL were completed.

After hypotonic treatment, cells were fixed in 3:1 methanol:acetic acid (Cinetica Quimica Ltda., Jandira, SP, Brasil/ Merck S.A Indústrias Químicas, Jacarepaguá, RJ, Brasil), suspended and centrifuged as previously described. After disposing supernatant, a fixation solution was added and cells were kept at 4 °C for one hour.

Hystologic slides were prepared with 2-3 drops of cellular suspension, stained with 0.5% Giemsa solution (Merck S.A) and analyzed using an optic microscope. Cell count was made using Paint® program.

RESULTS AND DISCUSSION

Most of the analyzed cell lines through electrophoresis showed the same LDH band number and migration patterns as the ones found in literature (ATCC, 1992). Primarily, differences on number of bands were noticed on low concentration extracts (1x10⁷) of NCTC clone 929, Caco-2, Vero, E.Derm, MDCK and NCI-H292 cells. More concentrated extracts of these cell lines (3-6x10⁷) were prepared, allowing us to visualize lighter bands, as described in literature for these species (ATCC 1992; Fernandes & Simoni 1995). A thinner teeth comb was used for better visualization and quality of assay (Figure 1).

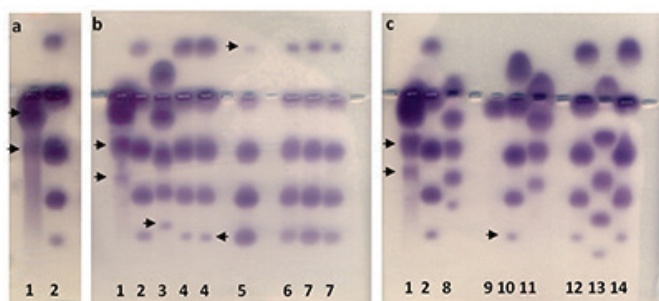


Figure 1: Horizontal LDH electrophoresis in agarose gel (a) Thicker teeth comb (2,0mm) low concentration extracts of NCTC clone 929 (1) and HeLa (2). Arrows indicate only two bands of NCTC clone 929. (b), (c) Thinner teeth comb (1,0mm) and more concentrated extracts (3 a 6x10⁷) of NCTC clone 929 (1), HeLa (2), E.Derm (3), NCI-H292 (4), Caco-2 (5), MRC-5 (6), LLC-MK2 (7), MDBK (8), BHK-21 (9), MDCK (10), SIRC (11), RD (12), PK(15) (13) and Vero (14). Arrows indicate presence of bands possessing lower expression which were observed only with higher concentrated extracts.

SIRC and MDBK cell lines didn't show the same LDH migration pattern as described in literature. The first band of both cell lines migrated to the negative electrode instead of the positive (Figure 1). This abnormality might be related to the different assay conditions, such as other types of buffers and gel as polyacrylamide, starch or the Authentikit™ system (Montes de Oca et al. 1969; ATCC 1992; Fernandes & Simoni 1995).

These adjustments allowed the visualization of lower expression bands without losing definition of higher expression bands.

These differences between the bands may difficult the identification since variations of expression are expected among several tissues of the same species (ATCC, 1992; Fernandes & Simoni, 1995; Nims & Herbstritt 2005).

Table 2 shows average and standard deviation of

each cell line migration distance. These data confirmed the reproducibility of the assay so that, in this condition, measurements of the distance between the bands will be used as standards for future cell lines authentication, it's made with the Authentikit™ system (ATCC 1992; Nims & Herbstritt 2005).

Table 2: Migration distance values of each LDH isoenzyme band (n=10).

Cell Lines	N° of bands (Authentikit system)	Migration distance averages (cm) - NCC-IAL					Standard Deviation (cm) - NCC-IAL				
NCTC clone 929	3	0,39	1,27	1,95	-	-	0,02	0,05	0,05	-	-
HeLa	5	-1,03	0,23	1,37	2,43	3,30	0,05	0,04	0,07	0,07	0,10
Caco-2	5	-1,04	0,25	1,38	2,46	3,45	0,05	0,04	0,04	0,06	0,10
RD	5	-1,07	0,23	1,34	2,40	3,32	0,08	0,03	0,08	0,09	0,12
NCI-H292	5	-1,03	0,26	1,37	2,39	3,35	0,04	0,05	0,04	0,07	0,07
MRC-5	5	-1,07	0,23	1,40	2,47	3,40	0,04	0,05	0,09	0,08	0,08
Vero	5	-1,13	0,18	1,30	2,37	3,31	0,08	0,06	0,07	0,10	0,12
LLC-MK2	5	-1,08	0,22	1,35	2,41	3,37	0,05	0,02	0,05	0,07	0,05
E.Derm	5	-0,42	0,62	1,51	2,38	3,09	0,04	0,07	0,05	0,04	0,05
MDCK	5	-0,71	0,27	1,47	2,49	3,36	0,11	0,03	0,10	0,10	0,10
MDBK	5	-0,20	0,67	1,35	2,04	2,66	0,02	0,06	0,05	0,07	0,08
PK(15)	5	-0,26	0,93	1,96	2,88	3,64	0,04	0,05	0,07	0,06	0,10
SIRC	4	-0,13	0,54	1,33	2,12	-	0,23	0,09	0,07	0,08	-
BHK-21	1	0,31	-	-	-	-	0,02	-	-	-	-

Table 3 shows number of metaphases and modal values obtained on karyotype. At least 50 metaphases of each cell line were analyzed as ATCC recommends (1994).

Table 3: Karyotype results, number of chromosomes of each species, quantity of analyzed metaphases and modal values of NCC-IAL compared to values of ATCC.

Cell Lines	Species	Regular chromosome number - 2n	Meta-phases number	Modal value	
				NCC-IAL	ATCC (1994)
HeLa	Human	46	50	82	82
RD			78	85	50
NCI-H292			60	48	47
Caco-2			50	95	96
MRC-5			50	46	46
NCTC clone 929	Mouse	40	170	66	66/67
Vero	African Green monkey	60	54	58	58
LLC-MK2	Rhesus monkey	42	56	65	65
SIRC	Rabbit	44	50	63	66
MDCK	Canine	78	51	79	78
MDBK	Bovine	60	50	52	51
PK(15)	Porcine	38	64	39	37
E.Derm	Equine	64	54	64	64
BHK-21	Syrian hamster	44	50	47	44

Most karyotype results show similarities with the ones in literature, however the RD cell line presented different values than the ones from ATCC. This variation might be due to differences on cultivation time, considering that ATCC used passage 34 (ATCC 1994) and in this study we used passage 340. This variation on chromosome number might occur at *in vitro* cell culture as the passage number increases and might interfere on cell chromosome stability (Li et al. 2009; Capes-Davis et al. 2010).

Although karyotype is a method that assists on cell species confirmation, depending on the transformation level of cell line, the chromosome number may vary. In this case, the use of banding as a complement of this technique is recommended. Electrophoresis provided quick, reproducible and lower cost results, proving to be a good alternative to commercial kit. Accuracy of results might increase further if two or more isoenzymes are used (Montes de Oca et al. 1979; Li et al. 2009).

Results obtained with the two methods are promising and facilitates the control and monitoring of cross contamination. It is important to emphasize that good laboratory procedures and the acquisition of certified cell lines from recognized banks are also essential to ensure quality and authenticity of cell lines.

ACKNOWLEDGMENT

We thanks Ms. Monaly Sado for cheking the use of English in the manuscript. Jéssica Zanoni Machado is a fellow of “Fundo Especial de Despesas do Instituto Adolfo Lutz” (FEDIAL).

REFERENCES

- American Type Culture Collection 1992. Quality control methods for cell lines. 2nd ed., ATCC, *Rockville*.
- American Type Culture Collection 1994. Catalogue of cell lines & hybridomas. 8th ed. ATCC, *Rockville*.
- Cabrera CM, Cobo F, Nieto A, Cortés JL, Montes RM, Catalina P, Concha A 2006. Identity tests: determination of cell-line cross-contamination. *Cytotechnology* 51: 45-50.
- Capes-Davis A, Theodosopoulos G, Atkin I, Drexler HG, Kohara A, Macleod RAF, Masters JR, Nakamura Y, Reid YA, Reddel RR, Freshney IR 2010. Check your cultures! A list of cross-contaminated or misidentified cell lines. *Int J Cancer* 127: 1-8.
- Dittmar KEJ, Simann M, Zghoul N, Schon O, Meyring W, Hannig H, Macke L, Dirks WG, Miller K, Garritsen HSP, Lindenmaier W 2010. Quality of cell products: Authenticity, identity, genomic stability and status of differentiation. *Transfus Med Hemother* 37: 57-64.
- Fernandes MJB, Simoni IC 1995. Caracterização de linhagens celulares. I - Identificação de espécies por análise isoenzimática. *Arq Inst Biol* 62: 59-63.
- Laboratory Procedures for Animal and Human Cell Lines. Reference n° AHC/1998/3/3.1/2.2. Accessed 14 March 2013. <http://www.cabri.org/guidelines/animal/AHC9833122.html>.
- Leandro M, Cruz AS 2012. Caracterização de Linhagens Celulares I. Verificação de espécie de origem. Análise dos padrões de migração da enzima LDH e cariótipo. *Bol Inst Adolfo Lutz* 22(2): 13-14.
- Li LF, Guan WJ, Li H, Bai XJ, Ma YH 2009. Establishment and characterization of a fibroblast cell line derived from Jining Black Grey goat for genetic conservation. *Small Ruminant Research* 87: 17-26.
- Losi CG, Ferrari S, Sossi E, Villa R, Ferrari M 2008. An alternative method to isoenzyme profile for cell line identification and interspecies cross-contaminations: cytochrome b PCR-RLFP analysis. *In Vitro Cell Dev Biol - Animal* 44: 321-329.
- Markovic O, Markovic N 1998. Cell cross-contamination in cell cultures: the silent and neglected danger. *In Vitro Cell Dev Biol - Animal* 34: 1-8.
- Milanesi E, Ajmone-Marsan P, Bignotti E, Losio MN, Bernardi J, Chegiani F, Soncini M, Ferrari M 2003. Molecular detection of cell line cross-contaminations using amplified fragment length polymorphism DNA fingerprint technology. *In Vitro Cell Dev Biol - Animal* 39: 124-130.
- Miranda ACS, Gonçalves CR, Ikeda TI, Cruz AS 2011. Padronização da técnica de eletroforese de isoenzimas para certificação de linhagens celulares. *Rev Inst Adolfo Lutz* 70(1): 77-80.
- Montes de Oca F, Macy ML, Shannon JE 1969. Isoenzyme characterization of animal cell cultures. *Proc Soc Exp Biol Med* 132(2): 462-469.
- Nardone RM 2007. Eradication of cross-contaminated cell lines: a call for action. *Cell Biol Toxicol* 23(6): 367-372.
- Nims RW, Herbstritt CJ 2005. Cell line authentication using isoenzyme analysis. Strategies for accurate speciation and case studies for the detection of cell lines cross-contamination using a commercial kit. *BioPharm International* 12(6). Accessed 01 April 2008. <http://www.biopharminternational.com/biopharm/article/articleDetail.jsp?id=166541>.
- Nims RW, Shoemaker AP, Bauernschub MA, Rec LJ, Harbel JW 1998. Sensitivity of isoenzyme analysis for the detection or interspecies cell line cross-contamination. *In Vitro Cell. Dev. Biol. - Animal* 34: 35-39.
- Ramya R, Nagarajan T, Sivakumar V, Senthilkumar RL, Bala Obulapathi B, Thiagarajan D, Srinivasan VA 2009. Identification of cross-contaminated animal cells by PCR and isoenzyme analysis. *Cytotechnology* 61(3): 81-92.
- Rojas A, Gonzalez I, Figueroa H 2008. Cell line cross-contamination in biomedical research: a call to prevent unawareness. *Acta Pharmacol Sin* 29(7): 877-880.
- Stacey GN, Bolton BJ, Doyle A 1992. DNA fingerprinting transforms the art of cell authentication. *Nature* 357:

261-262.

Steube KG, Grunicke D, Drexler HG 1995. Isoenzyme analysis as a rapid method for the examination of the species identity of cell cultures. In *Vitro Cell Devel Biol - Animal* 31(2): 115-119.

Wenger SL, Senft JR, Sargent LM, Bamezai R, Bairwa N, Grant SG 2005. Comparison of established cell lines at different passages by karyotype and comparative genomic hybridization. *Bioscience Reports* 24(6): 631-639.