The necessity of identity assessment of animal intestinal cell lines: A case report

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Abstract Eight intestinal cell lines, established from different animal species were submitted to DSMZ (German Collection of Microorganisms and Cell Cultures) in order to analyze their species of origin and their microbial contamination. Species identity was determined by PCR targeting mitochondrial genes and hence confirmed by sequencing the amplified PCR products. For three cell lines (CIEB, CLAB, PSI-1) we confirmed the species identity, whereas the species of origin of the three other cell lines (B6, B10XI and IPEC) was not the expected one: B6 and B10XI cells, which were supposed to be of chicken origin were identified as porcine cells. IPEC, allegedly a sub clone of the well-known porcine intestinal cell line IPEC-J2, was of bovine instead of porcine origin. However, two further IPEC-clones, namely IPEC-1 and IPEC-J2,

provided by another source were shown to be derived from the correct species (i.e. pig). Furthermore, six out of these eight cell lines turned out to be highly contaminated with mycoplasma. Alerted by this high incidence of infected and false specified cell lines, we feel obliged to inform all those working with animal intestinal cell lines and we strongly recommend verifying the species identity before using them. Also, the presence of mycoplasma should be tested when taking the cells in culture for the first time, and this mycoplasma control should be repeated at regular time intervals (e.g. every 4 weeks).

Keywords Cell Lines · Identity control of cell lines · Quality control of cell lines · Species-PCR · Speciation · Mycoplasma Infection · Intestinal cell lines · IPEC-cells

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Introduction

Continuous cell lines represent widely used models for a large number of life science research fields. Two basic requirements for the use of continuous cell lines in fundamental and applied research are their unequivocal identity and absence of contamination with microorganisms such as mycoplasma. Therefore, special emphasis must be taken (1) to detect and to prevent a microbial contamination and (2) to regularly monitor the identity of cell line(s) in use. The importance of quality control as an essential part of

good laboratory practice has been continuously addressed (Nelson-Rees et al. 1981; Stevenson 1987; Zoon 1993; MacLeod et al. 2002; Stacey et al. 2000; Masters 2002). Moreover, the recurrent reports of cross-contaminated and misidentified cell lines, mostly of human origin, underline the importance and necessity of identity testing (MacLeod et al., 1999; Drexler et al. 2003; Nardonne 2007; Lacroix 2008; Capes-Davis et al. 2010; Allston-Roberts et al. 2010).

In principle, cell lines can either be transformed or non-transformed, whereby the number of non-transformed, non-carcinogenic cell lines is widely underrepresented compared to tumor-derived ones. However, in the last few years a number of non-transformed human and animal cell lines have been proposed as model systems to study diverse aspects of intestinal physiology, toxicology and microbiology (Chantret et al. 1988).

Several animal intestinal cell lines are available from the investigating researchers and/or from public or commercial cell banks. Among these, especially two non-transformed animal cell lines, IPEC-1 and IPEC-J2, have been proven as valuable models studying gastrointestinal morphology and function (Gonzalez-Vallina et al. 1996; Schierack et al. 2006; Diesing et al. 2011; Nossol et al. 2011). Only very recently a functional and ultra structural characterization of IPEC-J2 was performed by Geens and Niewold (2011, this journal) emphasizing IPEC-J2 as a valuable in vitro model system for the intestinal epithelium.

In the present study the species of origin as well as the mycoplasma status of several animal intestinal cell lines from different laboratories were analyzed. The results show that it is imperative to determine the identity and the quality of intestinal cell lines of animal origin before the planned experiments are performed.

Materials and methods

Cell lines and culture conditions

CIEB, CLAB, IPEC, PSI-1, B6 and B10XI cells were first submitted from a third party to the Institute for Food Toxicology and Analytical Chemistry of the University of Veterinary Medicine (Hannover, Germany) and subsequently cultivated there, whereas

IPEC-1 and IPEC-J2 were deposited at the DSMZ cell line bank by Prof. Dr. Rothkötter, University of Magdeburg, (Germany). All cell lines were propagated in DMEM medium (high glucose) supplemented with 10–20% FBS, non-essential amino acids and in case of B6 and B10XI additionally with 140 µg/mL pituitary extract (Sigma-Aldrich, Steinheim, Germany).

Other cell lines, serving as standards (Table 1), were taken from the stock of DSMZ. Growth conditions and cell line characteristics of accessioned cell lines are described in the DSMZ Catalogue of Human and Animal Cell Lines (<http://www.dsmz.de>). Two samples of purified reference DNA from horse and donkey were obtained from Cibus Biotech (Rheda-Wiedenbrück, Germany).

PCR analysis and primer pairs

In order to minimize the risk of false PCR amplification, cell culture, DNA isolation, preparation of the reaction mix and final PCR were performed in different laboratories. PCR amplification and electrophoretic analysis of the products were described recently (Steube et al. 2003, 2008). Genomic sequencing of the PCR products was conducted by Eurofins

Table 1 Cell lines serving as controls

Cell line	Origin	Species ^a
BHK-21	Kidney	Syrian hamster
DT-40	Blood	Chicken
EBL	Lung	Bovine
FLK-BLV-044	Kidney	Ovine
GM-7373	Aorta	Bovine
HD-11 ^b	Blood	Chicken
HeLa	Cervix	Human
LAT	Aorta	Ovine
LLC-PK-1	Kidney	Pig
LMH ^b	Liver	Chicken
MDBK	Kidney	Bovine
MDCK ^b	Kidney	Dog
NIH-3T3	Embryo	Mouse
PK-15	Kidney	Pig
PC-12	Adrenal gland	Rat

^a Identified by Species-PCR (animals) or DNA fingerprinting (human)

^b Not available from the DSMZ cell bank; submitted to DSMZ only for internal use

Table 2 Primer pairs used in the Species-PCR

Primer name	5' to 3' sequence	Species	Target gene	Gene bank no.	Temperature (°C)
<i>Single and Duplex-PCR</i>					
Mito-Bov F1	gccatatactctccttggtgac	Bovine	ATPase 8/6	NC_006853	56
Mito Bov R1	gtaggcttggaatagtagcat	Bovine	ATPase 8/6	NC_006853	56
Mito Sus F	ctaaatctcccctcaatggtatg	Pig	ATPase 8/6	AF034253	57
Mito Sus R	gaatcctgtgaatacgggtgc	Pig	ATPase 8/6	AF034253	57
Mito-Chk F2	cgagtaatcatcaccgctgatga	Chicken	COII + ATPase 8	AP003580	60
Mito-Chk R2	gcttaggttcaggtcaggttca	Chicken	COII + ATPase 8	AP003580	60
Mito-Dog ^a	gaactaggtcagcccggtactt	Dog	COI	AY729880	60
Mito-Dog	cggagcaccaattattaacggc	Dog	COI	AY729880	60
<i>Multiplex-PCR</i>					
Sus ND F2	ctgctaattggatgatgacacg	Pig	NADH5	AF034253	58
Sus ND R2	ttcctgttaatgccaggcttc	Pig	NADH5	AF034253	58
Mito-Bov F2	atcgtactattccaagcctac	Bovine	ATPase 6	NC_006853	58
Mito Bov R3	ctgttaaccgcacggcga	Bovine	ATPase 6	NC_006853	58
Mito OV F	cgatacacgggttacttcacg	Sheep	COI	NC_001941	58
Mito Ov R	aaatacacgtcctattgataat	Sheep	COI	AF010406	58
Mito-Hrs F ^a	ctgcctaaagcctcctaata	Horse	COI	HM102300	58
Mito-Hrs R ^a	agaagtaggaatgatggggg	Horse	COI	HM102300	58

Column “target genes”, are the various mitochondrial genes analyzed: *ATPase 8/6* ATPase subunit 8 and ATPase subunit 6, *COII* cytochrome oxidase subunit II, *COI* cytochrome C oxidase subunit I, *NADH5* NADH Dehydrogenase subunit 5; the respective gene bank reference numbers are indicated

Last column “temperature” gives the annealing temperatures used in the individual PCRs

Mito mitochondrial (all sequences are from mitochondrial DNA) of the respective species given in the third column

^a Primer sequences were adapted from Cooper et al. 2007

MWG-Operon, Ebersberg, Germany. Oligonucleotide primers were obtained from Invitrogen, Darmstadt, Germany. Table 2 lists the applied primers for the identification of the animal species. Primers as well as method used for the mycoplasma detection were described earlier by Uphoff and Drexler (2011a).

Elimination of mycoplasma

Mycoplasma eradication was carried out by treatment with Baytril, Plasmocin and BM-Cyclin (in three independent subcultures) as previously described in detail (Uphoff and Drexler 2011b).

Results and discussion

The cell lines CLAB, PSI-1, IPEC (expected to be of porcine origin) and CIEB (expected to be of bovine origin) were sent from a third party to the University of Veterinary Medicine Hannover and subsequently

cultivated there. All cultures exhibited a low viability and a very poor growth. Initial testing revealed that they were contaminated by mycoplasma and therefore were submitted to DSMZ for both elimination of the bacteria and evaluation of the species of origin. At the DSMZ mycoplasma infection was confirmed by PCR, speciation revealed *Mycoplasma hyorhinis*, and the eradication of the cultures was started.

In parallel several PCR analyses were performed to determine the species origin using the specific primers given in Table 2. As controls several well-characterized and speciated cell lines from the DSMZ cell bank were included in the assays (Table 1).

The species origin of cell lines CLAB, CIEB and PSI-1 turned out to be as expected (Fig. 1a, b). In contrast, IPEC, a cell line related to the porcine intestinal cell line IPEC-J2, (Berschneider 1989), revealed a strong amplification product when bovine primer pairs were used (Fig. 1a), but none when porcine primer pairs were applied (Fig. 1b), strongly suggesting a false-specified origin (bovine instead of

Fig. 1 Analysis of genomic DNA from different cell lines using bovine (a) or porcine primer pairs (b). Amplified DNA fragments were detected after ethidium bromide staining of 1.2% agarose gels. DNA molecular weight markers of 500 bp and 1,000 bp are **bold**. a, primer pair: Mito-Bov. Lane 1: CLAB; lane 2: CIEB; lane 3: PSI-1; lane 4: IPEC; lane 5: PK-15 (pig, DSMZ ACC 640); lane 6: GM-7373 (bovine, DSMZ ACC 109); lane 7: water control (no DNA). Expected fragment size: 271 bp. b, primer pair: Mito-Sus. Lane 1: CLAB; lane 2: HELA (human, DSMZ ACC 57); lane 3: CIEB; lane 4: NIH-3T3 (mouse, DSMZ ACC 59); lane 5: PSI-1; lane 6: PC-12 (rat, DSMZ ACC 159); lane 7: IPEC; lane 8: PK-15 (pig, DSMZ ACC 640); lane 9: LAT (ovine, DSMZ ACC 349); lane 10: LLC-PK1 (pig, DSMZ ACC 637); lane 11: BHK-21 (hamster, DSMZ ACC 61); lane 12: water control (no DNA). Expected fragment size: 532 bp

porcine). These results were again verified, applying different sets of porcine and bovine primer pairs.

To finally clarify whether these IPEC cells were indeed of porcine origin or an incorrect species at source, two additional IPEC-samples, obtained from another laboratory, were analyzed. These two samples, IPEC-1 and IPEC-J2, have been intensively characterized in various studies before (Mariani et al. 2009; Pinton et al. 2010; Nossol et al. 2011; Dising et al. 2011).

As shown in Fig. 2, the two additional IPEC samples are indeed of porcine origin, this observation being in accordance with the results reported originally (Gonzalez-Vallina et al. 1996; Schierack et al. 2006) and supports the exclusion of a possible contamination during the course of the establishment of the original cell line IPEC-J2 (Berschneider 1989).

Amplification products of the cell lines IPEC-J2 and IPEC-1, as well as IPEC and CIEB and from the control cell lines PK-15 (porcine) and EBL (bovine, both DSMZ cell bank) obtained with bovine and porcine primers were purified and submitted to sequencing. The provided sequences were blasted and found to be 99% identical to published sequences for bovine mitochondrion in the case of EBL, CIEB, and IPEC and for porcine mitochondrion in case of

Fig. 2 Analysis of genomic DNA from different cell lines using a duplex-PCR with dog and chicken primer pairs. Amplified DNA fragments were detected after ethidium bromide staining of 1.2% agarose gels. DNA molecular weight marker of 500 bp is **bold**. Lane 1: B6; lane 2: B10XI; lane 3: MDCK (dog); lane 4: HELA (human, DSMZ ACC 57); lane 5: MDCK (dog); lane 6: NIH-3T3 (mouse, DSMZ ACC 59); lane 7: DT-40 (chicken, DSMZ ACC 636); lane 8: MDBK (bovine, DSMZ ACC 174); lane 9: HD-11 (chicken); lane 10: EBL (bovine, DSMZ ACC 192); lane 11: LMH-2 (chicken); lane 12: water (no DNA). Expected fragment size for dog: 154 bp and chicken: 474 bp

PK-15, IPEC-J2 and IPEC-1 cells, thereby again confirming that the species origin of IPEC was wrong.

Additionally, two other cell lines, B6 and B10XI, supposed to be derived from chicken intestine were analyzed. These two cell lines were also found to be heavily contaminated with *M. hyorhinitis* and again, the reputed species origin (chicken) could not be confirmed. No amplification products were detected after PCR analysis using chicken and dog (as a control) primer pairs (Fig. 2), whereas subsequent assays clearly revealed porcine signals for B6 and B10XI cells.

To summarize, a multiplex-PCR with bovine, ovine, porcine and horse primer pairs clearly confirmed once again that the species of origin of IPEC, B6 and B10XI cells (Fig. 3) was not the expected one (see also summary of the results in Table 3). Since the well-characterized IPEC-1 and IPEC-J2 cells, obtained from a different provider, were shown to be derived from the correct species, it can be deduced that the first examined IPEC sample, was mixed up or contaminated at a later stage of cultivation but not during original establishment of the IPEC-cell lines published by Berschneider (1989) and Gonzalez-Vallina et al. (1996).

Although it was not possible to obtain the cell lines B6 and B10XI from an alternative provider, a similar conclusion may be drawn for these two cell lines.

During the last years scientists at the DSMZ and other institutions have established easy-to-use methods for the analyses of animal cell lines (Stacey et al. 1997; Liu et al. 2003; Cooper et al. 2007; Ono et al. 2007; Steube et al. 2008). However, many cell lines,

Fig. 3 Analysis of genomic DNA from different cell lines using a multiplex-PCR with bovine, horse, ovine and porcine primer pairs. Amplified DNA fragments were detected after ethidium bromide staining of 1.2% agarose gels. DNA molecular weight markers of 500 and 1,000 bp are **bold**. Lane 1: IPEC-J2; lane 2: IPEC; lane 3: LAT (ovine, DSMZ ACC 349); lane 4: reference DNA horse; lane 5: IPEC-1; lane 6: CIEB (bovine); lane 7: FLK-BLV-044 (ovine, DSMZ ACC 153); lane 8: reference DNA donkey; lane 9: DT-40 (chicken, DSMZ ACC 636); lane 10: B6; lane 11: B10XI; lane 12: water (no DNA). Expected fragment sizes: porcine, 691 bp; bovine 415 bp; ovine 300 bp; horse/donkey, 244 bp

Table 3 Summary of identity and quality testing results

Cell line ^a	Species expected	Species identified	Mycoplasma infection
B6	Chicken	Pig	Yes
B10XI	Chicken	Pig	Yes
CIEB	Bovine	Bovine	Yes
CLAB	Pig	Pig	Yes
IPEC	Pig	Bovine	Yes
IPEC-1 ^a	Pig	Pig	No
IPEC-J2 ^a	Pig	Pig	No
PSI-1	Pig	Pig	Yes

^a Correctly specified and mycoplasma-free samples from the University of Magdeburg, Germany

especially animal cell lines may be shared between laboratories without further testing and the laboratories in which cell lines were established are often reluctant to deposit the cell lines in international cell repositories for quality assessments and further distribution (MacLeod and Drexler 2006). Despite repeated warnings there is still a high number of false or contaminated cell lines in use (Capes-Davis et al. 2010; Allston-Roberts et al. 2010) and waste of time and money due to invalid data can only be estimated (Stacey et al. 2000; Chatterjee 2007).

One may speculate that our findings are just an exception. However, based on the experience of DMSZ and the survey of the literature the presented cases most probably are the tip of the iceberg.

Therefore, it is strongly recommended that all laboratories working with cell lines, especially with animal cell lines, should take special care: the identity and quality of all cell lines should be examined before using them and repeatedly in regular intervals.

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