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BRIEF REPORT

Species identification and authentification of human and rodent cell cultures using polymerase chain reaction analysis of vomeronasal receptor genes

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Abstract Cell culture and the use of cell lines are routinely used in basic scientific research. It is therefore imperative for researchers to ensure the origin of the cell lines used and that they are routinely re-analysed for contamination and misidentification. Inter-species contamination is relatively frequent, and the most commonly used cell lines are of human, mouse and rat derivation. We have developed simple species specific primer assays based on genomic sequence differences in vomeronasal receptor gene family members to discriminate between human, mouse and rat DNA using standard agarose gel electrophoresis. Furthermore, these PCR assays are able to identify the species composition within an inter-species mixed population. This approach therefore provides a valuable tool to enable a rapid, simple and relatively inexpensive determination of the authentication and contamination of cell cultures.

Keywords PCR · Vomeronasal receptors · Species specificity · Genomic DNA

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Introduction

Cell lines which have acquired the ability to proliferate indefinitely are routinely cultured for use in laboratory studies. These continuous cell lines are repeatedly passaged, reliably cryopreserved and retain representative properties of the cells and tissues from which they were derived. These attributes have resulted in cell lines being widely used as model systems for the study of cellular processes in health and disease and are therefore commonly used within biomedical research and the pharmaceutical industry (Capes-Davis et al. 2010). Recently the robustness of cell cultures used in research has been under scrutiny as data have demonstrated that cross-contamination with other cells lines, misidentification of cell lines, and the use of cultures at high passage numbers has resulted in the generation of erroneous results (Hughes et al. 2007). Studies have highlighted the cross-contamination of various cell lines with well-established neoplastic cells. Indeed several human glioblastoma cell lines originally thought to be distinct have now either been shown to be identical or not of human origin (Chatterjee 2007; ECACC 2009). Moreover one of the first and most widely used human cell lines to be established, HeLa cells, has recently been demonstrated to have either contaminated many other cell lines or in many instances overgrown and replaced the original cells. A survey of 483 mammalian cell culturists indicated that 32% were unknowingly using HeLa cells whilst 9% were

using HeLa contaminated cultures. As a result 220 research reports listed in the PubMed database (1969–2004) were identified which had used HeLa contaminated cultures. One of the underlying problems responsible for this misrepresentation was that over a third of the researchers surveyed indicated that their cells had been derived from another laboratory rather than from a repository (Buehring et al. 2004). Subsequently these research issues have received significant media attention bringing into question the credibility of in vitro scientific studies and in particular the waste of public and private resource as a consequence (Northam 2007). Clearly it is therefore essential that due diligence is paid to ensure the integrity of cell cultures used for research purposes (Stacey 2000).

To aid researchers in identifying contaminated cell lines a database of cross contaminated or misidentified cell lines has been established (Capes-Davis and Freshney 2009). However it is also imperative to routinely monitor cell lines for possible contamination using characteristics that authenticate their identity. Over recent decades several methods have been established for use in authenticating and characterising cell lines, including allozyme and isoenzyme analysis (Stacey et al. 1997; O'Brien et al. 1980), karyotyping analysis (Rush et al. 2002), human leukocyte antigen typing (O'Toole et al. 1983), immunophenotypic and immunocytochemical analysis (Quentmeier et al. 2001), DNA fingerprinting and short tandem repeat profiling (Stacey et al. 1992; Dirks et al. 2005; Yoshino et al. 2006; Azari et al. 2007). In general these methodologies can be relatively time consuming and expensive as well as requiring experienced personnel to undertake them. Such features can make these techniques impractical for routine application.

Currently the majority of cell lines used in research and development are derived from human and rodents. Yet despite the burgeoning information present in the genome databases for these species only a handful of PCR-based technologies for cell line authentification have been developed (Stacey et al. 1997; Parodi et al. 2002; Liu et al. 2003; López-Andreo et al. 2005; Steube et al. 2003, 2008). Whilst these approaches may be relatively inexpensive many of these assays utilise multiple primers or require interpretation of relatively complex gel banding patterns. These factors can hamper their application and the transfer of the technique between laboratories. The aim of this present study was to use comparative genome sequence information for human, mouse and rat species to develop specific PCR assays which provide relatively simple, robust and unequivocal data for cell line identification and authentification.

Materials and methods

Cell lines and culture conditions

Mouse fibroblasts (3T3-Swiss) (Todaro and Green 1963) were cultured in Dulbecco's Modified Eagle Medium (DMEM) with 200 mM glutamine (Sigma, UK), 10% foetal calf serum (FCS) and 1% penicillin/streptomycin. Rat (WRC-256) (Hull 1953) and human (H400) (Prime et al. 1990) epithelial cells were cultured in DMEM/Ham's Nutrient Mixture F12 supplemented with 10% FCS and 1% penicillin/streptomycin or 0.5 μg/mL hydrocortisone, respectively. Primary bone marrow cells were isolated from 100 to 120 g male Wistar rats which were freshly sacrificed by cervical dislocation. Rat femurs were dissected, cleaned of excess soft tissue and repeatedly flushed with DMEM supplemented with 10% FCS and 1% penicillin/streptomycin (Sigma, UK). All cells were cultured in 25 cm^2 or 75 cm^2 flasks in a humidified incubator with 5% carbon dioxide in air at 37 °C. Medium was changed every 3 days.

Isolation of high molecular weight genomic DNA

All genomic DNA isolations from cells and tissues were performed using the PureLink kit (Invitrogen, UK) using the protocol recommended by the manufacturer. For initial processing of cultures (including cell lines and primary rat bone marrow cells), ~80% confluence cells were detached using trypsin/EDTA (Gibco, UK), cells were pelletted by centrifugation and washed twice with phosphate-buffered saline (PBS). For murine genomic DNA, 20 mg of liver was removed from an adult male Swiss mouse and processed by finely mincing prior to DNA isolation. For human genomic DNA isolation, heparinized (17 IU/mL) venous blood was obtained and red blood cells removed using erythrocyte lysis (0.83% NH_4Cl containing 1% $KHCO_3$, 0.04% $Na_2EDTA.2H_2O$ and 0.25% bovine serum albumin; 20 min). Isolated white blood cells were washed and re-suspended in PBS

supplemented with glucose (1 mM) and cations (1 mM $MgCl_2$, 1.5 mM $CaCl_2$). The cell suspension was pelletted by centrifugation and all supernatant removed prior to DNA isolation. Subsequently all cell pellets and dissociated tissue were resuspended in 200 μL of Genomic Digestion Buffer (Invitrogen, UK) and incubated for 10 min at 55 °C prior to genomic DNA isolation using the PureLink kit (Invitrogen, UK) using the protocol supplied by the manufacturer. DNA was eluted in 50 μL of buffer and concentrations were determined from absorbance values at a wavelength of 260 nm using a BioPhotometer (Eppendorf, UK). DNA integrity was also confirmed by visualisation on 0.8% agarose gel electrophoresis containing 0.5 μg/mL ethidium bromide (Helena Biosciences, UK). Samples were stored at −20 °C prior to use.

Genomic PCR amplification

Details of human and rodent oligonucleotide primers and cycling conditions used in this study are provided within the Table 1. Vomeronasal pheromone receptor gene sequences were obtained from GenBank and their specificity was confirmed by searches of human and rodent genomic sequence databases using the BLAST algorithm (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Primers were designed using Primer-BLAST software (http://www.ncbi.nlm.nih.gov/tools/primer-blast/index.cgi?LINK_LOC=BlastHome) and subsequently commercially obtained (Invitrogen, UK).

PCR assays were performed using the BioMix PCR system (Bioline, UK). Each reaction mix contained 50 ng of DNA in 1 μL of RNase free water, 12.5 μL BioMix Red, 9.5 μL dH_2O, and 1 μL each of 25 mM forward and reverse primers. PCRs were amplified in a Thermal Cycler (Mastercycler Gradient, Eppendorf) for 32 cycles. The programme consisted of an initial denaturation step for 5 min at 94 °C, followed by an amplification cycle consisting of 94 °C for 20 s, 20 s annealing at 66–67.5 °C (Table 1) and 68 °C extension for 20 s, ending with a final 10 min extension at 72 °C. Subsequently, 6 μL of each reaction was removed and the amplified product was separated and visualized under UV illumination on a 1.5% agarose gel containing 0.5 μg/ml ethidium bromide.

Results

Identification and validation of species specific PCR assays for cell line authentification

Recent publications have indicated that while pheromone receptors exhibit species conservation of gene sequence several family members are distinct to human, mouse and rat (Rat Genome Sequencing Project Consortium 2004; Shi et al. 2005; Grus et al. 2005). Subsequent BLAST analysis of the human, mouse and rat genomic sequences using selected vomeronasal receptor gene sequences retrieved from GenBank (Table 1) indicated their potential species specificity. Subsequently, for robustness, two primer

Table 1 Species specific genes and associated human and rodent primer assays

Gene name	Gene symbol	Species	Primer sequences (5′ → 3′)	Annealing temperature	Product size (bp)	Accession No.
Vomeronasal 1 receptor, 26	*V1rm1*	Rat	Forward: TGGCTTTCAGGCCACCAGGC Reverse: GCTCTGTCCTCAGGGGCAGGT	66.5 °C	387	NM001009525
Vomeronasal 1 receptor, 25	*V1rm2*	Rat	Forward: AGAAGAGTTACTGGCCCAAGGGACA Reverse: GGGGCTGAACGCTGGGAAGC	66.5 °C	408	NM 001008921
Vomeronasal 1 receptor, 200	*V1rh3*	Mouse	Forward: GGGAGGGGCCAGTGGCTACAT Reverse: TGCCACCAATCAACCAGAAGCCCA	66 °C	306	NM_134212
Vomeronasal 1 receptor, 210	*V1rh10*	Mouse	Forward: TTCAGGGTGCTATGGGAGGGGC Reverse: GCCCATCCCTGTGAATCAGCACA	66 °C	300	NM134235
Vomeronasal 1 receptor, 1	*VN1R1*	Human	Forward: TGGTCTGGGCCAGTGGCTCC Reverse: GAGTGTTTTCCTTGTCCTGCAGGCA	67.5 °C	332	NM_020633
Vomeronasal 1 receptor, 4	*VN1R4*	Human	Forward: TCGCACAGACACTGCGTGCA Reverse: ACACTGGGGTCACAGCTCATGAGA	67.5 °C	352	NM_173857

assays per species were designed to amplify products using relatively high annealing temperatures to enable specific DNA sequence binding (Table 1).

PCR analyses at a single cycle number (32) using genomic DNA derived from i) the cell lines, [WRC-256 (rat), Swiss 3T3 (mouse) and H400 (human)] and ii) control primary cells/tissues (rat bone marrow, mouse liver and human peripheral white blood cells) demonstrated that the assays designed amplified a specific product of the predicted size and that no cross species amplification was evident for any assay (Fig. 1). Data therefore indicated that in cell populations purely derived from a single species all assays supported culture clonality and authenticity.

Sensitivity of PCR assays for detection of mixed species populations

To determine whether the assays developed were able to detect mixed populations all combinations of species DNA were mixed at ratios of 5 ng : 45 ng and used for input in all PCRs. Data clearly demonstrated that with only a relatively minimal 10%/5 ng DNA inclusion all assays were robust and sensitive and able to detect minor contamination (Fig. 2).

Discussion

Several studies over the past decade have demonstrated the problem of cell line contamination which generally arises due to poor laboratory practice (Capes-Davis et al. 2010). Reported reasons include cell spreading via aerosols, use of unplugged pipettes, sharing media/reagents between cell lines and the use of mitotically inactivated feeder cells or conditioned medium, which have not had originating cells removed (van Pelt et al. 2003). Mislabelling, during either active culture handling or due to poor inventory records, is another source of misidentification (Capes-Davis et al. 2010). For future authentification and identification clear documentation of the cell line developed and robust assays are necessary for use in originating and recipient laboratories (Stacey 2000; Schmitt and Pawlita 2009).

Inter-species culture contamination is a common occurrence with the most frequently used cell lines being of human, mouse and rat origin (Steube et al. 2008). While assays based on karyotyping, fluorescent

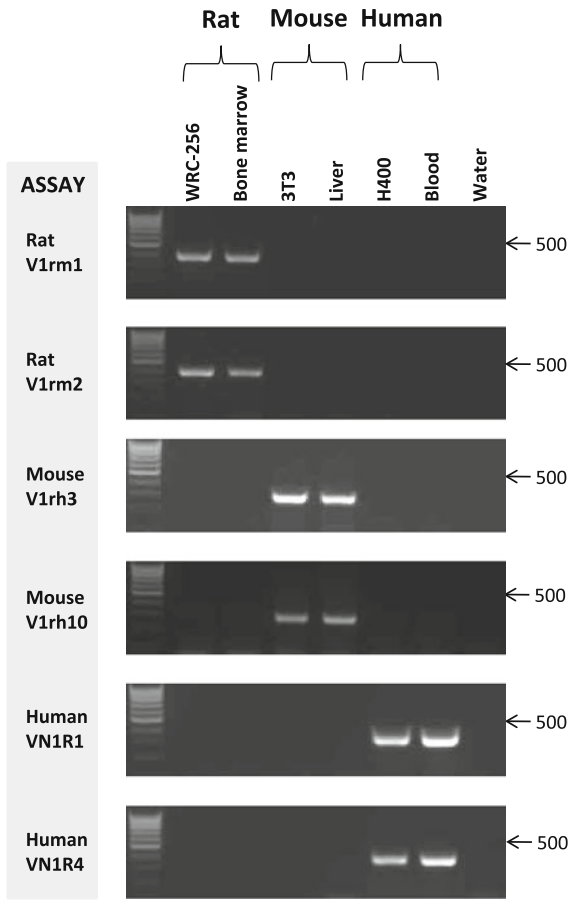

Fig. 1 PCR amplification with species specific primer pairs. PCR analysis of genomic DNA from rat, mouse and human cell lines and primary tissue using species specific primer pairs. PCRs assays used and source of input DNA (50 ng each) are shown. Amplified products generated at 32 cycles were detected by ethidium bromide staining of 1.5% agarose gels and UV transillumination. Blood refers to human peripheral white blood cells. RNase free water was used as negative control. *Arrows* indicate the DNA molecular weight marker of 500 bp (Hyperladder IV; Bioline)

in situ hybridization and isozyme analysis (Nardone 2007) have been developed to distinguish between cell lines from different species their application and interpretation can require significant expertise and costs which can inhibit their uptake. In-house approaches using relatively simple inexpensive PCR technologies are desirable however limitations remain with published approaches which can require complex gel banding pattern interpretation, multiple-primer/nested assays and/or application of complementary approaches for data confirmation. Many of the previous reports have also not demonstrated technique

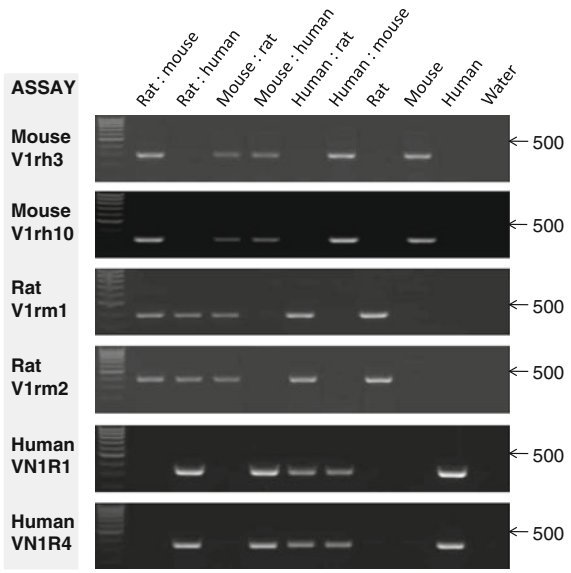

Fig. 2 Verification of the ability of the assay to detect interspecies cross contamination. DNA from each species was mixed at a ratio of 5 ng:45 ng (order as labelled) or used as 50 ng input from a single species. DNA used was: WRC-256 (rat); 3T3 (mouse); H400 (human). PCRs were performed with the primer assays indicated. Amplified products generated at 32 cycles were detected by ethidium bromide staining of 1.5% agarose gels and UV transillumination. RNase free water was used as negative control. *Arrows* indicate the DNA molecular weight marker of 500 bp (Hyperladder IV; Bioline)

sensitivity or ability to detect contamination (Liu et al. 2003; Ono et al. 2007; Ramya et al. 2009; Higgins et al. 2010; Parodi et al. 2002). Inexpensive assays for the identification and authentication of cell lines are therefore clearly necessary and should be relatively easily developed using the vast DNA databases as is demonstrated here. The simple assays reported here require minimal expertise for application and data interpretation and use standard molecular biology equipment. By combining exact amounts of DNA it was also able to sensitively detect minimal species contaminations.

Challenges still however remain for intra-species authentification and contamination detection. Techniques, such as genetic fingerprinting and immunocytological approaches can identify intraspecies contaminations as long as a known pure original stock is available as a comparator. It is however envisaged that high-throughput transcriptomic approaches may provide more robust future solutions which enable confirmation of the molecular phenotype of the cell lines providing assurances that they are representative of the tissue from which they were derived and are not contaminated.

The vomeronasal receptor PCR assays reported here provide a sensitive, accurate, rapid and cost effective approach for determining cell line authentification and inter-species contamination. In addition this test requires a relatively small number of cells, can utilise genomic DNA isolation kits from a range of suppliers and can easily be performed on a routine basis requiring only standard laboratory expertise.

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