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NONINVASIVE PCR SEXING OF NEONATAL RABBITS SELECTED FOR ISLET CELL CULTURE

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ABSTRACT

Fast PCR sexing of neonatal rabbits may be useful for rabbit gender management for breeding and research purposes. Buccal swabs were collected from 80 newborn rabbits and processed immediately. The buccal epithelial cells were simply lysed and used for fast PCR sex determination. Length difference of PCR products from male DNA samples allows fast and easy differentiation in agarose gel. PCR amplification on the buccal cell lysate DNA gave consistent and good results. All 80 tested rabbits were correctly sexed within 6 hours. The described method is fast, reliable and non-invasive for newborn rabbits.

Key words: genotyping; *Oryctolagus cuniculus;* H-Y antigen; sex determination; xenotransplantation

INTRODUCTION

Insulin dependent diabetes mellitus is a chronic, life-threatening disease characterized by a metabolic disorder in which there is a lack of action or production of insulin. Koblas *et al*. (2007) reported an attempt to identify a pancreatic endocrine progenitor and a stem cell, by employing genetic markers that are involved in the development of endocrine pancreas and β-cell neogenesis. They have shown one possible source of β-cells that could be easily obtained and, upon in vitro differentiation, give rise to insulin-producing cells. A major obstacle to providing whole pancreas or human islet transplantation to those in need is the lack of organs as they can only be obtained from cadaver donors. Therefore, the research in the diabetes field has focused on the identification of new sources of insulin-producing tissue.

Rabbit pancreas is an attractive source of islets. Rabbit insulin, like porcine, differs from human insulin at only one amino acid, and rabbit islets are glucose responsive (Reemtsma *et al.*, 1986 Giannarelli *et al.*, 1994). The large scale rabbit islet isolation has been developed by Hamelmann *et al.* (1994). The requirement of large number of male or female newborn animals for obtaining enough cells for establishing islet cell culture a rabbit gender management is therefore important for production of rabbits for research purposes. In addition, most human do not possess natural anti-rabbit antibodies, which might improve the possibility of preventing xenograft rejection (Forty *et al.*, 1992). Using newborn rabbit islets for xenotransplantation is described by Molnar (2006). Recently, the evaluation of intravascular xenotransplantation of macroencapsulated rabbit pancreatic islet cells in the process of the treatment of diabetes mellitus type 1 without immunosuppressive therapy has been described (Prochorov *et al*., 2008).

H-Y antigens which were originally discovered as transplantation antigens are the male-specific, Y chromosome–encoded minor histocompatibility (H) antigens (Simpson *et al.*, 1997a), others being encoded by autosomal genes. Antigenicity and allelism are created by sequence differences of peptides derived from intracellular proteins expressed at the cell surface

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following incorporation into major histocompatibility complex (MHC) class I and II molecules (Simpson and Roopenian 1997). This raises the possibility of modulating the immune response to grafts by inducing linked suppression via tolerance to immunodominant epitopes (Davies *et al.*, 1996). Clinical responses of patients undergoing female-to-male transplantation suggest that HY responses can be immunodominant over autosomally encoded minor H antigens in humans; thus induction of tolerance to HY antigens provides a model for inducing tolerance to autosomal minor H antigens via linked suppression. In view of this fact it is recommended female-to-female or male-to-male xenotransplantation of rabbit islet cell culture.

A simple, non-invasive technique for sexing newborn rabbits by eversion of the penis and vulva was reported by Fox and Crary (1972). It takes some practice, but it is even possible to sex newborn rabbits. Sexing of rabbits may seem like a simple thing to experienced breeders. In fact, even experienced breeders have problems in sexing newborn rabbits. Rabbit fetal sex determination by an anatomical description of the male and female gonads in the fetal rabbit during the last week of gestation has been reported by Nielsen and Torday (1983).

For the European rabbit and brown hare, a method for amplifying an SRY gene fragment together with a portion of the autosomal transferrin gene has been described (Wallner *et al*., 2001). Geraldes *et al*. (2005) sequenced 2388 bp of the European rabbit sex determining region Y (SRY) gene. Recently, Fontanesi *et al*. (2008) described a method for the sex determination of tissue originating from European rabbit, European brown hare and *Lepus timidus* based on PCR-RFLP analysis of point mutations that differentiate the ZFX and ZFY gene sequences. Buccal cells are a good source of DNA for animal genotyping (Wallner *et al*., 2001, Brooks *et al*., 2003, Meldgaard *et al*., 2004 Fontanesi *et al*., 2007).

Here we describe a fast, reliable and non-invasive PCR sexing method for neonatal rabbits which may be useful for rabbit gender management in production breeds or in research investigations.

MATERIAL AND METHODS

Buccal swab sampling and sample lysis

Samples were collected from 80 newborn rabbits of M91 (52) and P91 (28) rabbit lines (derived from NZW breed). Before that sampling rabbits were marked by permanent marker for easy identification after genotyping. For buccal cell sampling, widely available sterile cotton sticks were used to gently scrape the inner cheeks of the newborn animals according to Fontanesi *et al.* (2007) with some modification. Then the cotton sticks were cut an approx. 1cm from the cotton bud that was dropped in a 1.5 ml microcentrifuge tube. Samples were processed immediately to prevent bacterial growth and preserve the quality of DNA. Two hundred microliters of 1x PCR buffer (50 mM KCl, 20 mM Tris-HCl pH 8.4), supplemented with 50 mM DTT, 1% Triton X-100 and 0.4 µg/µl proteinase K was added to the tubes containing the cotton buds. The samples were briefly vortexed, incubated for 30 minutes at 55°C, and 5 minutes at 96°C and then cooled to room temperature. Samples were again briefly vortexed and centrifuged for 1 minute at 16060 x g. For PCR we used 1µl of the lysate.

As a control, rabbit genomic DNAs of 3 adult males and 3 females isolated from whole blood by salting-out method Miller *et al*., (1988) were used at the concentration of 50 ng per PCR.

PCR and sex determination

Duplex-PCRs were performed using primers amplifying a 241 bp fragment of the *Oryctolagus cuniculus* sex determining region Y (SRY) gene (AY785433) and a 487 bp fragment of the *Oryctolagus cuniculus* glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (L23961) (Table 1).

Table 1: Primers used for the sex determination analysis

Primer name	Primer sequence from 5' to 3'	nt position	GenBank Acc. No.	PCR product
OcSRY21F	AGCGGCCAGGAACGGGTCAAG	736-756	AY785433	241 bp
OcSRY23R	CCTTCCGGCGAGGTCTGTACTTG	976-954		
OcGAPDH21F	TGAACGGATTTGGCCGCATTG	91-111	L23961	487 bp
OcGAPDH21R	ATGCCGAAGTGGTCGTGGATG	577-557		

The two-step PCR conditions (PTC-200 DNA Engine; MJ Research) were 98°C for 30 s, 92°C for 1s, 64°C for 35s, 35 cycles with last extension at 72°C for 1 min. The reaction volume (15µl) contained 20 mM $(NH_4)_2SO_4$, 75 mM Tris-HCl pH 8.8 (at 25°C), 0.1% (w/v) Tween 20, 1.5 mM $MgCl_2$, 0.4 µM of each primer, 250 µM dNTPs, and 1U Platinum Taq DNA Polymerase (Invitrogen).

The amplified DNA was electrophoretically separated on 2% agarose gels containing ethidium bromide at 180 mA in 10mM lithium borate buffer, pH 8.0 for 10 minutes. The products were visualized under UV light and photographed using a GDS 8000 (UVP) camera (Figure 1).

RESULTS AND DISCUSSION

The described assay co-amplified a 241 bp fragment of the rabbit SRY and a 487 bp long fragment of the autosomal rabbit GAPDH gene, which is served as internal amplification control. To verify the reliability of the PCR method, the genomic DNA isolated from whole blood of adult males and adult females was also amplified in each PCR assay (Figure 1).

After the genotyping newborn rabbits were grouped according to PCR sex determination, and at the age of three weeks the sex of the animals was controlled by penis and vulva eversion according to Fox and Crary (1972). The morphological assessment was in total agreement with the molecular results.

Several methods have been described using buccal cells as a source of DNA for genotyping. Wallner *et al.* (2001) employed them for rabbit and hare sex determination, Brooks *et al.* (2003) for comparative animal genomic analysis, Meldgaard *et al.* (2004) for PCR analysis of transgenic mice, and Fontanesi *et al.* (2007) for rabbit analysis of MC1R gene mutations. All these methods used the non-invasive sampling by harvesting buccal epithelial cells from swabs for subsequent cell lysis or direct PCR.

The cotton buds may stay in the tubes after buccal cell lysis because they did not affect the release of DNA into lysis buffer and its subsequent amplification. The buccal swabs lysate can be stored for up to one week at room temperature without loss of performance in the PCR sexing assay (not shown).

The buccal cells lysate provided both sufficient quantity and quality of amplifiable DNA and thus can be used for several rabbit genotyping rounds, e.g. to screen different genetic markers such as mutations in the rabbit MC1R gene (Fontanesi *et al*., 2007).

The described PCR protocol run in a standard thermocycler is completed after 54 minutes. Duplex PCR allowed omitting the time-consuming restriction digestion of PCR products as required in other molecular sexing methods (Fontanesi *et al*., 2008).

The length difference in 246 bp between PCR products of SRY and GAPDH from male DNA samples allowed an easy differentiation of amplified DNA fragments in agarose gels. The use of lithium borate based electrophoresis buffer (modified from Brody and Kern, 2004) permits to perform electrophoresis at 180 mA for rapid fragment resolution in 2% agarose gel at room temperature (Figure 1).

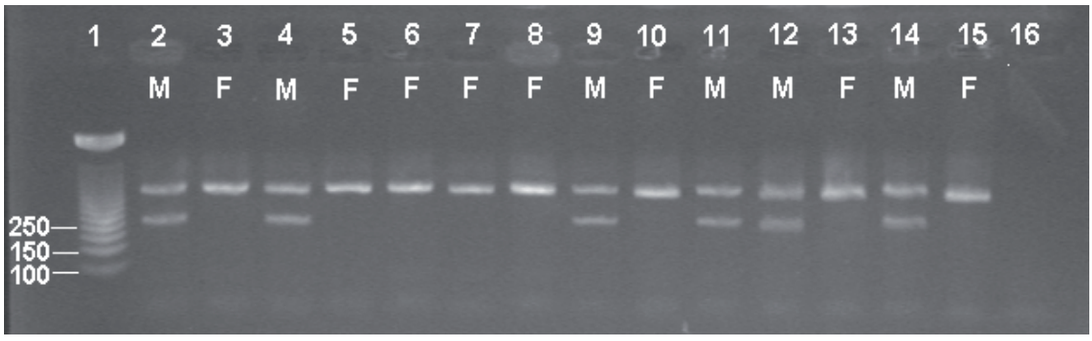

Fig. 1: Representative results of fast PCR sexing of newborn rabbits. In all samples PCR products of rabbit GAPDH (487 bp) were amplified as internal amplification control. Lane 1: molecular weight marker: 50 bp DNA Step Ladder (Promega, G4521). Lanes 2, 4, 9, 11, 12, 14: amplified the rabbit SRY specific fragment of 241 bp, indicating males. Lanes 3, 5, 6, 7, 8, 13, 15: in females only the internal control PCR product (487 bp) was amplified. Lane 14: adult male rabbit genomic DNA. Lane 15: adult female rabbit genomic DNA. Lane 16: non-template control. M=male, F=female.

At our conditions sex determination for 80 newborn rabbits was performed within 6 hours. A single buccal epithelial cell lysate was sufficient for at least 20 PCR reactions.

In conclusion, buccal swabs were collected from neonatal rabbits and processed immediately. The buccal epithelial cells were simply lysed and used for fast PCR sex determination by SRY specific fragment. The buccal cell lysate provided both sufficient quantity and quality of amplifiable DNA.

To enhance efficiency by minimizing PCR cycling time and thus maximizing throughput we shortened the PCR profile to the two-step protocol. Amplified fragment of the autosomal rabbit GAPDH gene is served as internal amplification control. The described method is fast, reliable and non-invasive for neonatal rabbits. This method has the advantage of the simplicity of the methods of specimen collection that are easier than blood or other type of tissue sampling.

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