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Determination of Sex and Scrapie Resistance Genotype in Preimplantation Ovine Embryos

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ABSTRACT The aim of this study was to test the accuracy of genotype diagnosis after pre-amplification of DNA extracted from biopsies obtained by microblade cutting of ovine embryos and to evaluate the viability of biopsied embryos after vitrification/warming and transfer to recipients. Sex and PrP genotypes were determined. Sex diagnosis was done by PCR amplification of ZFX/ZFY and SRY sequences after PEP-PCR while PrP genotype determination was performed after specific pre-amplification of specific target including codons 136, 154 and 171. Embryos were collected at Day 7 after oestrus. Blastocysts and expanded blastocysts were biopsied immediately after collection whereas compacted morulae were biopsied after 24 hr of in vitro culture. Eighty-nine biopsied embryos were frozen by vitrification. Fresh and vitrified whole embryos were kept as control. DNA of biopsies was extracted and pre-amplified. Sex diagnosis was efficient for 96.6% of biopsies and PrP genotyping was determined in 95.8% of codons. After embryo transfer, no significant difference was observed in lambing rate between biopsied, vitrified control and fresh embryos (54.5%, 60% and 66.6%, respectively). Embryo survival rate was not different between biopsied and whole vitrified embryos ($P = 0.38$). At birth, 96.7% of diagnosed sex and 95.4% of predetermined codons were correct. Lamb PrP profiles were in agreement with parental genotype. PEP-PCR coupled with sex diagnosis and nested PCR coupled with PrP genotype predetermination are very accurate techniques to genotype ovine embryo before transfer. These original results allow planning of selection of resistant genotype to scrapie and sex of offspring before transfer of cryopreserved embryo. *Mol. Reprod. Dev.* © 2008 Wiley-Liss, Inc.

Key Words: biopsy; dpi; PrP genotyping; sex diagnosis; vitrification; transfer

similar to bovine spongiform encephalopathy (BSE) in cattle, Creutzfeldt-Jacob disease (CJD) and kuru in humans, or chronic wasting disease (CDW) in wild ruminants. TSEs are characterized by the accumulation of an abnormal isoform (Protein Prion Scrapie, PrP^{Sc}) of the normal cellular prion protein (Protein Prion Cellular, PrP^C) in the central nervous system of affected animals, according to the prion hypothesis (Prusiner, 1982). This pathogenic conformer is insoluble, protease K resistant (Bolton et al., 1982) and had a higher content of β -sheet (43%) as compared to the normal form of PrP^C (3%) (Pan et al., 1993). Sheep resistance and susceptibility to scrapie is largely under genetic control, particularly the prion protein (PrP) gene (Dickinson, 1976; Hunter, 1997). Major polymorphisms of this gene are located at codons 136 (T, A, V), 154 (R, H) and 171 (R, Q, H, K), but only some combinations were found: ARQ, a probable ancestral allele, VRQ, AHQ, ARH, ARR, 4 frequently derived alleles, and rarely derived alleles TRQ and ARK. The ARR and AHQ haplotypes are mainly associated with resistance and VRQ with susceptibility (Hunter et al., 1996; Elsen et al., 1999; Diaz et al., 2005). Recently, new codons associated with scrapie resistance were detected (Goldmann et al., 2005). In several European breeds, selection of the ARR/ARR genotype has been implemented to improve resistance to scrapie, mainly due to possible links between animal diseases and human health. This selection was carried out by crossing parents of resistant genotype and only ARR/ARR rams were conserved. Little is known about transmission of scrapie between animals or flocks but maternal transmission to lambs is strongly suspected. The most likely pathway of scrapie transmission from ewes to lambs is an early

INTRODUCTION

Scrapie is a fatal transmissible spongiform encephalopathy (TSE) in sheep and goats caused by prions,

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lateral post-natal event rather than in utero transmission (Andréoletti et al., 2002). Thus, embryo transfer procedures could circumvent vertical transmission even when the offspring are of a high-risk genotype (Wang et al., 2002).

The biopsy of early mammalian embryos is now commonly used for genotyping and assessment of certain inherited genetics disorders (for a review see Bredbacka, 2001; Georges, 2001). The challenge is to detect by marker assisted selection (MAS) genetic information linked to economic trait loci to implement in breeding program. In particular, sex predetermination of offspring allows sex ratio manipulation in domestic animals and has a major impact on animal breeding, decreasing the generation interval and the cost of the breeding process. Since the 1990s, polymerase chain reaction (PCR) was largely used to amplify a Y chromosome-specific sequence (SRY, sex-determining region Y) either alone, or with an autosomal sequence for control (Peura et al., 1991; Bernardi and Delouis, 1996; Lopes et al., 2001; Manna et al., 2003; Mara et al., 2004). Genes common to X and Y chromosomes (ZFY/ZFX, zinc finger protein) can also be amplified by a single primer pair and after amplification, a specific restriction endonuclease is required to identify male and female (Aasen and Medrano, 1990; Bredbacka and Peippo, 1992; Bredbacka et al., 1995; Kochhar et al., 2000). The number of isolated cells affects the accuracy of PCR sex diagnosis. When fewer than five cells were analysed, the efficiency of sexing decreased (Lacaze et al., 1996, 2007). Accordingly, 8–10 blastomeres have been recommended for successful sexing of embryos in the field (Thibier and Nibart, 1995). A novel DNA amplification method, loop-mediated isothermal amplification (LAMP), without thermal cycling and electrophoresis, was reported to sex embryos efficiently with only 3–5 cells (Notomi et al., 2000; Hirayama et al., 2004).

When several genetic markers are to be analysed, sufficient DNA is required to obtain an accurate result. However, biopsy can be detrimental to embryo viability and should be minimized in order to avoid pregnancy failure after embryo transfer. Therefore, preliminary amplification of the genome from only a few cells is needed to obtain sufficient DNA for several genetic analyses without reducing embryo viability. A primer extension pre-amplification (PEP) was described by Zhang et al. (1992) for the amplification of the whole genome from a single cell using a random mixture of 15-base oligonucleotides as primers. Variable efficiency of this technique was reported in regard to the length of the PEP program, 30–100% (Xu et al., 1993; Hochman et al., 1996; Sermon et al., 1996; Chrenek et al., 2001). Another approach was to preamplify by PCR a genomic fragment including all regions of interest (nested PCR) before the specific PCR amplification (Appa Rao and Totey, 1999; Lien et al., 1999; Virta et al., 2002; Peippo et al., 2007). All these pre-amplification techniques take time and the biopsied embryos must generally be stored by cryopreservation before transfer, waiting for result of genotyping diagnosis.

In the present study, sex diagnosis and PrP genotype predetermination were carried out on ovine embryo microblade biopsies. Sex diagnosis was done by PCR amplification of ZFY/ZFX and SRY sequences after primer extension pre-amplification (PEP-PCR), while PrP genotype determination was done after specific pre-amplification (nested PCR) of specific sequence including codons 136, 154 and 171. Accuracy of genotype diagnosis after DNA pre-amplification was tested and the viability of biopsied embryos was evaluated after vitrification/warming and embryo transfer.

MATERIALS AND METHODS

Animals

The oestrous cycles of Ile-de-France and Aragonese ewes (donors and recipients) were synchronized by insertion of an intravaginal sponge containing 40 mg FGA (Chronogest[®], Intervet, Angers, France) for 13 days (Day 0 = sponge insertion). At sponge removal, the recipients received 500 IU of eCG (Chronogest[®]). Donors were superovulated with a total of 200 µg pFSH (Merial, Belgium) injected intramuscularly every 12 hr in six decreasing doses (50, 50, 30, 30, 20 and 20 µg) during the last 3 days of progestagen treatment (Days 11–13). pFSH treatment was supplemented with 66 and 90 µg LH in the last two injections, respectively. Donors were mated with selected rams and/or inseminated in utero under endoscopic control with frozen/thawed semen from selected fertile rams (200 × 10⁶ spermatozoa per female) 20–24 hr after the onset of oestrus. The mating was chosen in order to obtain large variability of embryo PrP genotype.

Embryo Collection

Embryos were recovered surgically by laparotomy under general anesthesia (i.v. thiopental 10 mg kg⁻¹, atropine sulphate 0.3 mg kg⁻¹ and endotracheal intubation with isoflurane and oxygen) 7 days after the onset of estrus. Each uterine horn was flushed with 40 ml of prewarmed PBS containing 0.2% bovine serum albumin (BSA) (Euroflush, IMV, l'Aigle-France). The embryos were immediately retrieved and placed in PBS containing 0.4% BSA (Embryo holding medium, IMV, l'Aigle-France) at +20 to +25°C. They were selected according to their quality (grades 1 and 2) and classified according to their stage of development. Blastocysts and expanded blastocysts were selected for biopsy (0 hr biopsy). Compacted morulae were in vitro cultured for 24 hr in modified synthetic oviduct fluid (SOF; Holm et al., 1999) with 5% foetal calf serum (FCS) in 5% CO₂, 5% O₂, 90% N₂ atmosphere before biopsy at the blastocyst stage (24 hr biopsy). Whole blastocysts and expanded blastocysts were vitrified as controls at 0 and 24 hr. For fresh whole embryo transfer, only compacted morulae, blastocysts and expanded blastocysts were kept. After embryo collection, local antibiotic (Aluspray) was sprayed on the abdominal incision, and each donor received benzylpenicilline and dihydrostreptomycine for 3 days (i.m., 1 g per day).

Embryo Biopsy

Prior to biopsy, each embryo was washed 3 times in a 30 μ l drop of PBS without BSA. The biopsy was carried out using an inverted microscope (CK-2; Olympus, 200 \times) and a Narishige micromanipulator with a metal microblade adapted as a cutting instrument. The microblade was positioned above the embryo, taking care to exclude the inner cell mass and include only the trophectoderm. A small piece composed of approximately 8–10 cells of trophectoderm was cut with a single vertical movement of the microblade. After cutting, a microdrop of PBS with 0.4% BSA was added. Trophectoderm cells of the biopsy were detached from the piece of zona pellucida and were deposited in a sterile tube without medium and frozen at -20°C until DNA extraction. The biopsied embryo was immediately vitrified, with or without zona pellucida. Between each embryo biopsy, the microblade was washed in water, then 70% ethanol, water again and finally PBS without BSA. This biopsy technique permitted the sectioning of 15–20 embryos per hour.

Embryo Cryopreservation

Biopsied embryos were individually loaded in each straw, while two whole embryos were loaded per straw for direct transfer. The vitrification method described by Mermillod et al. (1997) and modified by Guignot et al. (2006) was applied. Briefly, embryos were kept at room temperature for 5 min in PBS supplemented with 20% new-born calf serum (NBCS). They were then vitrified in three steps at room temperature as follows: 10% glycerol for 5 min, 10% glycerol and 20% ethylene glycol (EG) for 5 min, and finally 25% glycerol, 25% EG and 0.4 M sucrose for 30 sec in PBS-NBCS. During the last step, embryos were quickly aspirated into the centre of a 0.25 ml plastic straw (IVM, L'Aigle, France) within 20–30 μ l of vitrification solution. Embryos were separated by two air bubbles from two surrounding segments

of PBS-NBCS containing 0.8 M galactose (about 90 μ l each). The straws were sealed and immediately plunged into liquid nitrogen.

DNA Extraction

DNA of biopsies was extracted at 35°C for 30 min with proteinase K. The extraction mix (20 μ l) contained 2 μ l of $10\times$ PCR buffer, 0.6 μ l of 10 $\mu\text{g}/\mu\text{l}$ proteinase K (Sigma, P-2308) and 17.4 μ l of water. Proteinase K was then neutralized at 98°C for 10 min before pre-amplification.

DNA from blood of rams, ewes and lambs was extracted using standard alkaline lysis method.

Primer Extension Pre-Amplification (PEP-PCR) and Sex Diagnosis on Embryo Biopsy

Whole genome amplification by PEP-PCR was performed as previously described (Zhang et al., 1992). Briefly, to 3 μ l of extracted and neutralized samples was added 1.6 μ l of a 400 μM solution of 15-base random oligonucleotides (Sigma–Aldrich, Saint Quentin Fallavier, France), 1.2 μ l of 25 mM MgCl_2 , 0.2 μ l of 10 mM dNTPs, 2 μ l of $10\times$ PCR buffer, 0.3 μ l of Taq polymerase (Eurogentec, 5 U/ μ l) and brought to 20 μ l with water. After a 4 min DNA denaturation step at 94°C , 50 primer extension cycles were carried out in a gradient thermocycler (Mastercycler[®] gradient, Eppendorf, Germany). Each cycle consisted of a 1 min denaturation step at 94°C , following by a 2 min annealing at 37°C , a programmed ramping step of $0.3^{\circ}\text{C}/\text{sec}$ to 55°C and a 4 min polymerase extension step at 55°C . Each sample was then analysed for sex diagnosis.

Duplex PCR was carried out to amplify the ZFX/ZFY gene (Zinc Finger Protein), gene common to X and Y chromosomes, and the SRY gene (sex-determining region Y), a Y chromosome-specific sequence. Primer sequences and GenBank accession numbers of targets are given in Table 1. Five μ l of pre-amplified sample were

TABLE 1. Primers Sequences

Gene	GenBank accession no.	Primer	Sequence (5'–3')
ZFX/ZFY	AJ000269/AJ000270	P1-5EZ	ataatcacatggagagccacaagct
		P2-5EZ	gcacttcttggatctgagaaagt
SRY	Z30265	SRY-c-F1	gctgggatacagagtggaaaa
		SRY-c-R1	agctgctgtgatgctccttt
PRNP	DQ408530	PrePRP-F	ccagtaagcaaaaaaccaa
		PrePRP-R	agcctgggattctctctggt
		PRP1	aagtgtactacagaccagttgatc
		PRP2	atgcacatttgcaccactctgc
		PRP5	gcagctggagcagtggttaggg
		PRP171F	gttaccacaacaaagtgtactacaga
		PRP171R	tgttgacacagtcagcacaag
		PRP154F	tggcaatgactatgaggaccg
		PRP154R	tggtctgtagtacacttggtggg
		PRP136F	gatagtaacggctcctcatagtcattgc
		PRP136R	ctgcagctggagcagtggtta
		Probe171-R	FAM-ccagctggatcggtata-Q
		Probe171-G	VIC-accagctggatcagata-Q
		Probe171-H	VIC-accagctggatcattat-Q
		Probe154-R	FAM-actatcgtgaaaacat-Q
		Probe154-H	VIC-tactatcatgaaaacatg-Q
		Probe136-A	FAM-tcatggcacttcc-Q
		Probe136-V	VIC-ctcatgacacttcc-Q

added to 1.2 µl of 25 mM MgCl₂, 0.4 µl of 10 mM dNTPs, 0.4 µl of each 25 µM primer, 2 µl of 10× PCR buffer, 0.2 µl of Taq polymerase (Eurogentec, 5 U/µl) and brought to 20 µl with water. The PCR protocol included an initial step of 94°C for 4 min, followed by 35 cycles of 94°C for 20 sec, 60°C for 20 sec, 72°C for 40 sec and ended with a final extension at 72°C for 5 min. All PCR products were visualized by ethidium-bromide-stained 2% agarose gel electrophoresis. After migration of PCR products, males have two bands at 320 bp (SRY) and 447 bp (ZFX/ZFY), and females only one band at 447 bp (ZFX).

Specific PrP Gene Pre-Amplification

PCR was performed to amplify a 369 bp region of the PrP gene of including codons 136, 154 and 171 with specific primers PrePRP-F and PrePRP-R given in Table 1. Five microlitres of extracted and neutralized samples were added to 1.2 µl of 25 mM MgCl₂, 0.4 µl of 10 mM dNTPs, 0.4 µl of each 25 µM primer, 2 µl of 10× PCR buffer, 0.2 µl of Taq polymerase (Eurogentec, 5 U/µl) and brought to 20 µl with water. The PCR protocol included an initial step of 94°C for 4 min, followed by 36 cycles of 94°C for 20 sec, 63°C for 20 sec and 72°C for 40 sec and ended with a final extension at 72°C for 5 min. Product identity was confirmed with ethidium-bromide-stained 2% agarose gel electrophoresis before PrP genotyping. PCR-RFLP and TaqMan[®] assays were applied to determine PrP genotype of the biopsy.

PrP Genotyping

PCR-RFLP and TaqMan[®] assays were applied to determine PrP genotype of the biopsy, ewes, rams and lambs. Primers and probe information are given in Table 1.

PCR-RFLP genotyping was conducted as in Elsen et al. (1999), with slight modification for primer PRP5. Briefly, genotype at codon 171 was determined by the restriction profile of a PCR fragment obtained using primers PRP1 and PRP2 with *Bcl*I enzyme. Genotype at codons 136 and 154 was determined by the restriction profile of a PCR fragment obtained using primers PRP5 and PRP2 with *Bsp*HI enzyme.

TaqMan[®] genotyping assay was also used to genotype separately codons 136, 154 and 171: each codon was determined by a 5' nuclease assay with specific F and R primers for amplification, and TaqMan probes with FAM or VIC fluorochromes and Quencher coupled to allele-specific oligonucleotides, on ABI PRISM 7900 HT (Applied Biosystems, Courtaboeuf, France).

Warming and Embryo Transfer Procedures

Transfers were done at the end of the reproductive season (winter), in two different locations (CITA Zaragoza Spain and INRA Nouzilly France). After warming, embryos were transferred directly into synchronized ewes, 7 days after the onset of oestrus detected with vasectomised rams 24–48 hr after sponge removal. Two embryos were transferred per recipient. Recipients were multiparous and dry ewes maintained indoors for 3 months before transfer. They were

sedated with 0.3 mg kg⁻¹ i.v. acepromazine (Calmivet[®], Vetoquinol, Lure, France). After endoscopic control of the presence of at least one functional corpus luteum, embryos were transferred as follows. For warming, straws were held 5 sec in air followed by 15 sec in a 22°C water bath. After warming, the entire contents of the straw (250 µl) was deposited surgically with a prototype instrument (IMV, l'Aigle-France) into the uterine horn without embryo selection nor cryoprotectant removal. For fresh whole embryos transfer, two embryos were put, just after collection, into a Tomcat linked to a 1 ml syringe and introduced into the uterine horn of the recipient.

Pregnancy Rate

Pregnancy rate was diagnosed at Day 17 after estrus by progesterone assay (RIA method) (Saumande and Thimonier, 1972): a female was considered pregnant if progesterone concentration was higher than 1.5 ng/ml. It was confirmed by ultrasound examinations on Day 42 and PAG assay (pregnancy-associated glycoprotein; RIA method) (Gonzalez et al., 1999) on Days 42 and 90, and finally by observation of lambing at term. The lambing rate was defined as the lambed/transferred recipient ratio and the embryo survival rate was defined as the lambs born/transferred embryo ratio.

Parentage Control

At birth, parentage control of each lamb was performed to assess clearly which transferred embryo had given a product. After DNA extraction from blood, genotypes were identified using a panel of 9 microsatellite markers (CSRD0247, OLADRB, INRA49, MAF214, MAF65, McM42, McM527, OarFCB20, TGLA53). Allele sizes were determined by capillary electrophoresis on ABI3100 automated sequencer (Applied Biosystems). Parentage was controlled by checking the mendelian transmission of alleles from parents to the products.

Statistical Analysis

This study was designed to compare different embryo qualities after transfer, biopsied versus control, and vitrified versus fresh. Comparisons were performed using the Chi-square test. Statistical significance was denoted as $P < 0.05$.

RESULTS

Embryo Collection and Biopsy

Sixteen donors were synchronized for embryo biopsy. From 4 donors, only unfertilised oocytes or retarded embryos were recovered ($n = 37$). From the 12 other donors, 110 transferable embryos (i.e. compacted morulae and blastocysts) and 44 unfertilised or retarded embryos were recovered. Among the 110 transferable embryos, 20 were kept for control and 90 were biopsied. During embryo biopsy, one biopsy was lost and the corresponding embryo was eliminated. Among the 89 embryos, 71 were biopsied and vitrified just after in vivo collection and 18 after 24 hr of in vitro culture.

Among the 20 whole embryos kept for control, 16 were vitrified just after collection and 4 after culture.

Sex Diagnosis and PrP Genotype Predetermination on Embryo Biopsy

After primer extension pre-amplification (PEP-PCR) and sex diagnosis, sex genotyping of 86 embryos was clearly diagnosed (96.6%), and 3 were thought to be female but it was uncertain. Results of PrP genotype determination are given in Table 2. Among the 89 embryo biopsies, 76 PrP genotypes were efficiently determined (85.4%). Ten embryo biopsies had two possible genotypes (one principal and one considered as contamination), and three had an incompatible genotype between the two techniques and remained undetermined for 2 and 4 codons out of 6. Among 534 analysed codons, 512 were determined (95.8%). When parent genotype was compared, 76 embryo biopsy genotypes were concordant (85.4%) (70/76 genotyped +5/10 contaminated +1/3 not fully determined embryo biopsy), six were impossible, five had 2 possible genotypes and two remained not fully determined. Comparison with parent genotype allowed determination of 515 codons out of 534 (96.4%).

Lambing and Embryo Survival Rate

All 89 biopsied embryos were transferred to 45 recipients (one recipient received just one embryo). Eight days after transfer, one recipient died and was excluded from the study. No significant differences were found between locations or recipient genotype (Ile de France or Rasa Aragonesa) so, the results were pooled. The rate of pregnancy obtained for biopsied and vitrified control embryos are shown in Table 3. For biopsied and vitrified control embryos, results of pregnancy were lower when embryos were cultured 24 hr before vitrification compared to 0 hr vitrification, but the difference was not significant and results were pooled. The pregnancy rate, evaluated at Days 17, 42, 90 was not significantly different between each group of transfer and the lambing rate was not affected by embryo biopsy and embryo vitrification, 54.5%, 60% and 66.6% respectively for biopsied embryos, whole vitrified embryos, and fresh whole embryos. The embryo survival rate was not significantly affected by biopsy when embryos were vitrified ($P = 0.38$), but tended to be lower when biopsied and vitrified embryos were compared to fresh whole

embryos (34.5%—30/87 vs. 52.6%—20/38, respectively, $P = 0.057$).

Accuracy of Sex Diagnosis and PrP Genotype Determination

Among the 30 lambs coming from biopsied, sexed and transferred embryos, 29 (96.7%) had an accurate sex diagnosis. Only one male was diagnosed as a female. The recipients which received an embryo suspected to be female but uncertain were not pregnant. One lamb died 1 month before term and unfortunately no blood sample was taken, so descendant control and PrP genotype analysis were carried out on just 29 lambs. Accuracy of PrP genotype determination is given in Tables 2 and 4. Among the 70 determined PrP genotypes corresponding to parent genotype, 25 transferred embryos gave birth and all 25 PrP genotype predeterminations were accurate. Among the 5 double genotypes (contaminated genotype), one embryo (B) gave birth and the right PrP genotype was the principal one (not the contaminant). Among both not fully determined genotypes, two lambs were born (C, D). Lamb PrP genotype was in agreement to parent PrP genotype and to partial embryo biopsy genotype (4 codons among 6). Finally, among 6 impossible PrP genotype determinations, one lamb was born (A): lamb PrP genotype was in agreement with parent genotype, but not with embryo biopsy genotype for 2 codons among 6. Altogether, on 174 determined codons (6×29 born lambs), 166 (95.4%) were accurately predetermined.

DISCUSSION

In the present study, sex diagnosis and PrP genotype determination were carried out on ovine embryo microblade biopsies. Accuracy of genotype diagnosis and viability of the biopsied embryos were tested after vitrification, warming and embryo transfer into recipient. The embryo survival rate obtained with biopsied/vitrified embryos was similar to those reported by Leoni et al. (2000) (34.5% vs. 40%) in the same species. This rate was not significantly different to that obtained with whole vitrified embryos in the present study. Only 10.5 points were lost after embryo microblade biopsy (34.5% for biopsied vitrified embryos versus 45% for whole vitrified embryo). A greater loss, 16%, was reported by Hasler et al. (2002) with in vivo frozen bovine embryos and the same technique of biopsy (50% vs. 66%), but only 8% with fresh embryos. Quality and

TABLE 2. PrP Genotype Determination on Embryo Biopsy, Comparison With Parent Genotype and Control With Lamb Genotype After Birth

Embryo biopsy genotype, number of determined codons	Parents genotype comparison, number of codons in agreement	Lamb genotype control (29 births), number of accurately determined codons
76 genotyped (85.4%), 456/456	70 concordant (78.7%), 420/420 6 impossible (6.75%), 30/36	25 exact genotypes (86.2%), 150/150 1 wrong (lamb A) (3.45%), 4/6
10 contaminated (11.2%), 46/60	5 concordant (5.6%), 30/30 5 contaminated (5.6%), 23/30	0 1 exact (lamb B) (3.45%), 6/6
3 not fully determined (3.4%), 10/18	1 determined and concordant (1.1%), 6/6 2 not fully determined (2.25%), 6/12	0 2 exact (lambs C and D) (6.9%), 6/12

TABLE 3. Pregnancy Rate of In Vivo Produced Sheep Embryos, After Biopsy and Vitrification

Embryo	Time of vitrification	Recipient n (embryos)	Pregnancy rate % (n)					Embryo survival	Lamb sex
			Day 17	Day 42	Day 90	Lambing			
Biopsied vitrified	0 hr	36 (71)*	69.4 (25)	61.1 (22)	58.3 (21)	58.3 (21)	38 (27)	14 f, 13 m	
	24 hr	8 (16)	62.5 (5)	37.5 (3)	37.5 (3)	35.5 (3)	18.75 (3)	1 f, 2 m	
	Total	44 (87)	68.2 ^a (30)	56.8 ^a (25)	54.5 ^a (24)	54.5 ^a (24)	34.5 ^a α (30)	15 f, 15 m	
Control vitrified	0 hr	8 (16)	62.5 (5)	62.5 (5)	62.5 (5)	62.5 (5)	50 (8)	4 f, 4 m	
	24 hr	2 (4)	100 (2)	50 (1)	50 (1)	50 (1)	25 (1)	1 m	
	Total	10 (20)	70 ^a (7)	60 ^a (6)	60 ^a (6)	60 ^a (6)	45 ^a α β (9)	4 f, 5 m	
Fresh		21** (38)	85.7 ^a (18)	66.6 ^a (14)	66.6 ^a (14)	66.6 ^a (14)	52.6 ^a β (20)	8 f, 12 m	

f: female; m: male.

a, b values differ significantly ($P < 0.05$ chi-square test).

α , β tendency ($P = 0.057$ chi-square test).

*One recipient had received just one embryo.

**Four recipients had received just one embryo.

development stage of the embryos have a real effect on the embryo viability after microblade biopsy, especially after cryopreservation. Peippo et al. (2007) have obtained the highest number of excellent quality Day 8 embryos for transfer with advanced developmental stages and embryo of quality code 1 after bovine embryo microblade biopsy. Biopsy size could be reduced and then embryo viability enhanced. The good result of embryo survival obtained in the present study with blastocysts and expanded blastocysts demonstrated that microblade biopsy technique could be a field method of choice for ovine blastocysts, with poor embryo damage even if a few cells are destroyed around the cutting edge and the surrounding zona pellucida is frequently completely removed with this technique compared to cell aspiration.

Embryo survival rates obtained with fresh and vitrified control embryos observed in this study were not significantly different (52.6% and 45%, respectively). These results were similar to those found by Alabart et al. (2007) with fresh embryos (55.8%), but they are somewhat lower than those reported previously by Baril et al. (2001) (60% and 50% with fresh and vitrified embryos, respectively), Leoni et al. (2000) and Dattena et al. (2004) (68.7% and 63%, respectively, with vitrified embryo). This difference could be attributed to batches and breeds of recipient females, vitrification technique or age of donors and season of embryo production that may affect embryo quality.

After PEP-PCR and sex diagnosis, percentages of correct sex diagnosis obtained in the present study are

very high: 96.6% of samples were efficiently diagnosed, and 96.7% of born lamb sex was accurately diagnosed. These results are similar to those of Hochman et al. (1996), Chrenek et al. (2001) and Tominaga and Hamada (2004) with the same method of genome pre-amplification in bovine (97%, 91% and 89%, respectively) and to those of Mara et al. (2004) with ovine biopsied embryos, but without pre-amplification (100% efficiency and 87% accuracy). In the present study, one male was diagnosed female. Even without whole genome pre-amplification, it is the common mistake. However, as ZFY/ZFX gene was used as an autosomal control, a specific restriction endonuclease could be applied on top of this to totally ensure the correct sex of ovine embryo biopsy (Bredbacka and Peippo, 1992; Bernardi et al., 1996). Despite this mistake, the present study reported for the first time a sex diagnosis method on embryo biopsy coupled with whole pre-amplification genome giving very encouraging success rates in sheep, without increasing contamination risk due to several steps required. Moreover, it generated a lot of copy of the whole genome, which allowed different multiplex genotyping on the same sample. However, as nested PCR was noticed to be more efficient and accurate than PEP-PCR for PrP during preliminary tests, nested PCR was chosen instead of PEP-PCR to test for the first time PrP genotype predetermination on ovine embryo biopsy.

After nested PCR, the percentage of predetermined PrP genotype from 89 embryo biopsies was acceptable; 85.4% of biopsies could be completely predetermined and 95.8% of codons were predetermined. The accuracy

TABLE 4. PrP Genotype of Lambs A, B, C and D, Their Parents and Corresponding Embryo Biopsies

Lambs	Lamb genotype	Parent genotype	Embryo biopsy genotype
A	AHQ/VRQ	ARR/VRQ, AHQ/ARQ	VRQ/VRQ
B	ARQ/AHQ	ARR/ARQ, AHQ/ARQ	<u>ARQ/AHQ</u> , contaminated by <u>ARR/ARQ</u>
C	ARR/ARR	ARR/VRQ, ARR/ARR	?R?/?R?
D	ARQ/AHQ	ARR/ARQ, AHQ/ARQ	<u>AR?/AH?</u>

Underlined codons of embryo biopsies genotype are in agreement with parent genotype. Codons written in bold are wrong predetermined or undetermined.

of PrP genotyping was high too (95.4%). These results are in agreement with those of Virta et al. (2002) and Peippo et al. (2007) in bovine embryos with the same pre-amplification method (nested PCR), but with another target gene region (ZFX/ZFY and milk production markers), 96–99% of accuracy. Few incorrect determinations of PrP genotypes are probably the consequence of preferential amplification of one allele, or limited efficiency of PCR on very small samples.

It is obvious that genotyping or sex determination may have imperfect efficiency or reliability when the amount of DNA is very limited. The performance in the present study was comparable with other similar studies performed using very small DNA samples. All these studies working on low quantities of DNA share basically the same process: a first step of genome or target amplification, and a second step of genotyping. Both steps have specific risks. PEP-PCR may lead to incomplete genome coverage (portions of the genome can be lost), and produce usually small fragments of DNA (at each cycle, degenerate primers bind internally to previous products). New whole genome amplification procedures, as multiple displacement amplification (MDA, a non PCR-based method), can provide a greater yield of DNA from tiny samples, with longer fragments, and could be less prone to allele dropout (Dean et al., 2002; Hellani et al., 2004; Sun et al., 2005; Peng et al., 2007). To take into account the relatively small size of DNA fragments produced by genome amplification (PEP-PCR or MDA), it is possible to make smaller PCR that could be more efficient for sex determination or genotyping using specifically designed primers. For PrP genotyping, two different techniques were used in the present study: PCR/RFLP and TaqMan[®] assays, and only 3 samples out of 89 gave partially conflicting results between these two techniques. Usually, TaqMan[®] genotypes had very high confidence score because it is more sensitive than PCR/RFLP, but both techniques can be affected by bias in genome amplification, and particularly allele dropout. It is difficult to circumvent allele dropout in such cases. To increase the confidence level in the genotypes, it is possible to replicate genome amplification and genotyping on different aliquots of DNA extracts (multiple tubes approach, as in Taberlet et al., 1996).

Even if more studies are needed to try to reach similar results with PEP-PCR or other techniques of whole genome amplification as with nested PCR, these totally original results on ovine scrapie predetermination from embryo biopsy, followed by embryo cryopreservation, are very encouraging. It will increase the efficiency and reduce the cost of selection process aiming to eradicate alleles of scrapie sensitivity in the ovine population.

CONCLUSIONS

DNA pre-amplification, PEP-PCR and nested PCR are very accurate for sex diagnosis and PrP genotype predetermination, respectively, from ovine embryo microblade biopsy. Post transfer viability of *in vivo* biopsied embryos after vitrification/warming tended to

be slightly reduced compared to intact vitrified or fresh embryos. These original results allowed selection at the same time genotypes resistant to scrapie and sex of offspring before transfer of cryopreserved embryos. Moreover, other target genes linked to other diseases or to production traits could be analysed on the same biopsy sample thanks to PEP-PCR conferring an increased economical value to the embryo obtained by multiple ovulation embryo transfer (MOET).

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