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Application of Whole Genome Amplification Technique for Genotype Analysis of Bovine Embryos

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***Abstract*—**In recent years, there has been an increasing interest toward the use of bovine genotyped embryos for commercial embryo transfer programs. Biopsy of a few cells in morulla stage is essential for preimplantation genetic diagnosis (PGD). Low amount of DNA have limited performing the several molecular analyses within PGD analyses. Whole genome amplification (WGA) promises to eliminate this problem. We evaluated the possibility and performance of an improved primer extension preamplification (I-PEP) method with a range of starting bovine genomic DNA from 1-8 cells into the WGA reaction. We optimized a short and simple I-PEP (ssI-PEP) procedure (~3h). This optimized WGA method was assessed by 6 loci specific polymerase chain reactions (PCRs), included restriction fragments length polymorphism (RFLP). Optimized WGA procedure possesses enough sensitivity for molecular genetic analyses through the few input cells. This is a new era for generating characterized bovine embryos in preimplantation stage.

***Keywords*—**Whole genome amplification (WGA), Genotyping, Bovine, Preimplantation genetic diagnosis (PGD)

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I. Introduction

RESEARCH in embryo technologies has been gradually focused on the preimplantation genetic diagnosis (PGD) [1]. The genotyping of bovine embryos for quantitative traits locus (QTL) marker haplotypes as well as major QTLs which have been distributed overall genome in preimplantation stage have a high importance for animal breeding companies. Marker/gene assisted selection (MAS/GAS) projects can be performed in preimplantation stage through mass production of characterized embryos. Limited quantity of cells (1-2 biopsied blastomers) and low amount of DNA is the main challenge. Initial amplification of whole genome of biopsied cells was proposed for overcoming this limitation. Whole genome amplification (WGA) is an in-vitro method to produce a large quantity of DNA from minimal genomic DNA sample [2].

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Although, conventional PCR may have an enough sensitivity to perform DNA analysis in a single cell, a single cell can be analyzed only once. Furthermore independent confirmation and additional analyses are impossible [3]. Primer extension preamplification based on PCR (PEP-PCR) is the first WGA technique which uses the completely degenerate (random) primers and Taq DNA polymerase in low stringency thermal cycles. It is simulated that at least 99.8% of the genomic sequences in a diploid cell is amplified at least 30 times [4]. The optimization of PEP, known as improved-PEP-PCR (I-PEP-PCR) method, proposed aims to increasing the amplification efficiency from single cells. The use of DNA polymerase mixture which one of them has the proofreading activity leads to a further improvement in the sensitivity of the method, this is due to the more processivity of the polymerases with 3'-5' exonucleous activity [3], [5], [6]. Therefore the extension products of these polymerases are longer. Several primer-extension (PE) and thermal-cycling based WGA methods were next proposed, which all of them utilize either definitive or semidegenerate primers [7]. Random primers are able to prime on templates that are as complex as the human genome [8], [9], and with these primers it is not necessary to have the previous information about target genome sequence, moreover the product of WGA techniques which utilize such primers is an unbiased representation of template genomic DNA [5].

Isothermal amplification methods (e.g. multiple-strand displacement amplification or MDA) are not based on repetitive cycles of denaturation and annealing [10], [11]. These methods proposed for overcoming the limitation of PCR-based WGA methods such as incomplete genome coverage and short products; however the flaws were indicated later [12]. The comparison between I-PEP-PCR and MDA suggested that the amount of specific product of I-PEP is more than of MDA [4]. Most of the researches performed around WGA techniques have been focused on human genome, therefore in this study we assessed the applicability and sensitivity of an optimized short and simple procedure based on I-PEP-PCR for the whole genome amplification of bovine cells.

II. Materials and Methods

The genomic DNA (gDNA) was extracted from male and female Holstein blood samples and/or embryos (in morrula or early blastocyst stages). DNA samples extracted from blood either were serial diluted or diluted up to DNA amount in 1 (~6pg μl^{-1}), 2, 4 and 8 diploid cell.

TABLE I
CHARACTERISTICS OF THE PRIMERS

aim	name	sequence	chromosome	annealing tem. (ºC)	restriction enzyme
control	1.715	5' TGG AAG CAA AGA ACC CCG CT 3' 5' TCG TCA GAA ACC GCA CAC TG 3'	satellite	63	-----
sexing	BRY.4a	5' CTC AGC AAA GCA CAC CAG AC 3' 5' GAA CTT TCA AGC AGC TGA GGC 3'	Y	63	-----
	Y	5' CCT CAG CTG CTT GAA AGT TC 3' 5' GAT CTG TAA CTG CAA ACC TGG 3'	Y	63	
genotyping	kappa-CSN	5' GCT GAG CAG GTA TCC TAG TTA T 3' 5' CTT CTT TGA TGT CTC CTT AGA G 3'	6	50	HindIII
	GH	5' GCT GCT CCT GAG GGC CCT TCG 3' 5' GCG GCG GCA CTT CAT GAC CCT 3'	5 & 19	67	AluI
	PRL	5' CGA GTC CTT ATG AGC TTG ATT CTT 3' 5' GCC TTC CAG AAG TCG TTT GTT TTC 3'	23	58	RsaI

After washing the embryos within the phosphate-buffer-saline (PBS) droplets containing polyvinylpyrrolidone (PVP, 1µg µl^{-1}) and removing the Zona-pellucida (ZP) using pronase solution (5%), the blastomers were dissociated by slowly pippeting in trypsin solution (0.25%) droplets. One, 2, 4 and 8 blastomers immediately were transferred into PCR tube containing 9µl enzymatic lysis buffer (PCR mixture containing 0.2µg µl^{-1} proteinase K). Activation and inactivation of proteinase were performed through incubation in 65ºC and 98ºC, respectively. In the first experiments, we followed the original I-PEP protocol [6]. We optimized then a short and simple procedure based on I-PEP. We used Pfu DNA polymerase as polymerase which possesses proofreading activity in reactions.

Purification of extension products was performed using silicagel column. WGA reaction were performed in a 25µl reaction mixture containing gDNA (6-48pg µl^{-1} or 1-8 cell), 10pM 15-mer random primers (estimated contains 10^9 different sequences), 4×200µM dNTPs, 1X PCR buffer, 1.5mM $MgCl_2$, 5% DMSO and 2µl DNA polymerase mixture pfu:Taq (with the ratios 1:10, 1:7 and 1:3 in the original I-PEP, ssI-PEP and specific reactions, respectively). The sequence of primers utilized for locus-specific PCRs (sexing and genotyping) using the product of initial WGA reaction and restriction enzymes used for restriction fragments length polymorphism (RFLP) in genotyping step are given in table I.

III. RESULTS AND DISCUSSION

The electrophoresis of I-PEP reaction products based on original protocol and using 4 serial diluted DNA samples (until 0.0001ng µl^{-1}) produced a smear, mostly greater than 2kb in size (Fig. 1). This confirms that the amplification of whole genome has been performed successfully. Moreover there was not a significant difference in products size or intensity between samples containing various amounts of template DNA. This concurs with the results of others [10], [13], [14].

The original I-PEP protocol is very time-consuming (~13-14 hours); on account to that the WGA step is just an initial step to provide more starting material for subsequent main analyses and the time between biopsy and transfer of desirable embryo is critical factor, so it is important to decrease the necessary time as possible as for first step. We observed that the ramping step (0.1ºC Sec^{-1}) between annealing and extension steps in the original protocol has an obvious effect on the length of primer extension in WGA reaction.

Additionally, since the primers used in PE based WGA methods are short and completely degenerate, they can prime to internal regions of previous cycles products. Therefore, the shorter fragments will produce with each additional cycle. Such products are not suitable for subsequent specific analyses [15]. Furthermore, polymerase will become a limiting factor after almost 30 cycles, this is due to the existence of numerous annealing sites for random primers [16]. On account to these problems, we optimized a short and simple procedure through the elimination of ramping step from the original I-PEP method and reduction of the cycle number to 35 (instead of 50). Another change was the increasing of Pfu ratio in DNA polymerase mixture (1:7 instead of 1:10 for Pfu:Taq in the original protocol). These changes decreased the required time for whole genome amplification into <3h.

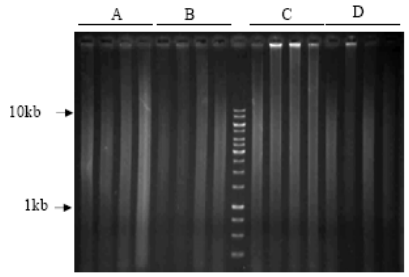

Fig. 1 Gel electrophoresis pattern of WGA products for 4 diluted DNA samples until 0.0001ng µl^{-1}

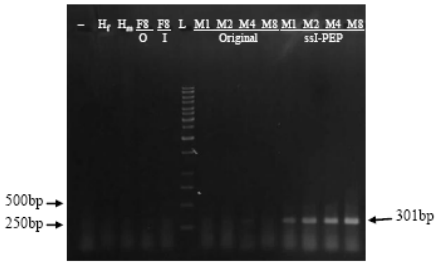

Fig. 2 Gel electrophoresis pattern of specific PCR with primer BRY.4a; H, F, M: human, female and male samples, respectively; Original: samples without initial WGA; ssI-PEP: samples after ssI-PEP WGA procedure; L: 1kb ladder; 1-8: cell count; -: negative control

We considered that the performing locus specific PCRs using wgaDNA as template is the most suitable way for evaluation of each WGA method. We performed the specific analyses in two categories included sexing and genotyping. We entered 5µl aliquots of ssI-PEP product from samples which had DNA amount equivalent to 1, 2, 4 and 8 diploid cells in specific PCRs with primer BRY.4a. Correct 301bp band was observed *only* for male samples that initially subjected to whole genome amplification (lanes 11-14 in Fig. 2). This confirms that the optimized ssI-PEP procedure has enough sensitivity for specific analysis on the few cells. Fig. 2 represents that, as input cell number within WGA reaction increase specific band becomes more severe.

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The correct embryos sexing in preimplantation step is an important factor, therefore we considered another sex specific sequence for definitive and reliable conclusion. Furthermore, since there is a sufficient starting material (wgaDNA), it is possible to allocate several specific PCRs using different primers on one same sample. It was a sequence of bovine Y-locus and the expected fragment (308bp) was amplified in male few cell samples only after initial whole genome amplification (Fig. 3).

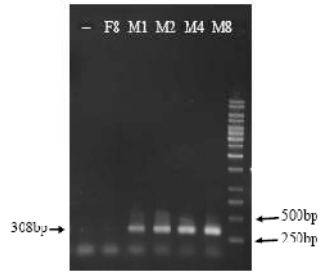

Fig. 3 Gel electrophoresis pattern of specific PCR with primer Y on ssI-PEP product; F, M: female and male samples, respectively; 1-8: cell count; -: negative control

The amplifying several bovine specific sequences is an internal control,truly, however we are able to consider an autosomal repetitive sequence like bovine 1.715 into another reaction using wgaDNA of one sample. It is for ensuring about that the WGA product is not from contamination and/or non-bovine origin. In this case 216bp band should be observed in both male and female samples. When we used DNA extracted from 1-8 cells without initial WGA reaction into specific analyses, there was not distinguishable specific band and/or specific band was hardly distinct (data not shown).

Sex determination of embryos is just one of the several possible PGD analyses using WGA product. In this study, we assessed the sensitivity and reliability of optimized WGA procedure for amplifying 3 important QTL markers in dairy cattle breeding programs which allocate on different chromosomes (Table I): kappa-casein (kappa-CSN), prolactin (PRL) and growth hormone (GH). Using 5µl aliquots of optimized procedure product in specific reactions resulted to the amplify 443bp, 223bp and 156bp fragments for kappa-CSN, GH and PRL loci, respectively, as we expected. When we used 2µl (instead of 5µl) WGA product and/or twice diluted wgaDNA on 2 and 4 cell samples, the correct 443bp fragment also obtained with kappa-CSN primer (Fig. 4). This is an important result; with the use of lesser amount of WGA product we will be able to perform genetic analyses within as more as possible loci and to obtain more complete genetic profile for embryos in preimplantation stage.

Determination of genotype for QTL markers was performed by digestion of amplified specific fragments with relevant restriction enzyme (e.g. Fig. 5 for prolactin locus). The comparison between genotype determined with wgaDNA and gDNA of same sample showed that there is a concordance except of 2 cases. There was a mismatch between genotype determined with wgaDNA and gDNA, only for 2 samples with kappa-CSN primer; heterozygous (AB) with gDNA (contain 116.4 and 181.8 ng μl^{-1} DNA) whereas homozygous (BB) with wgaDNA on 2-cell samples. For example, the digestion results for one of these two samples represents in Fig. 6 and Fig. 7. Lane 7 in Fig. 6 represents the electrophoresis pattern after digestion of amplified kappa-CSN fragment on the original gDNA (contained 116.4 ng μl^{-1} DNA), whereas lane 5 in Fig. 7 represents the electrophoresis pattern after digestion of amplified kappa-CSN fragment on wgaDNA of the same sample, which had been diluted until 2-cell DNA amount (12pg μl^{-1} DNA). This mismatch is due to the preferential amplification of one of the kappa-CSN alleles (allele B), and in other word allele drop-out (ADO) for another allele (allele A), in which there is not restriction site for HindIII.

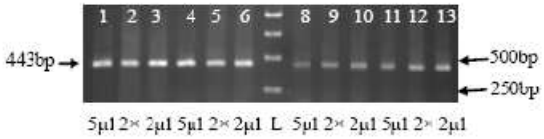

Fig. 4 Gel electrophoresis pattern for specific PCR with primer kappa-CSN on 2 and 5µl and twice diluted (2×) of ssI-PEP products from different 2 (lanes 1-3 and 8-10) and 4 (lane 4-6 and 11-13) input cells, L: 1kb ladder

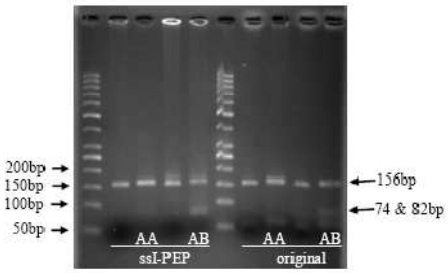

Fig. 5 Gel electrophoresis pattern obtained before and after digestion of amplified prolactin fragment on 2-cell samples subjected to WGA (ssI-PEP) and same original samples (contain 456.4 and 106.4 ng μl^{-1} DNA).

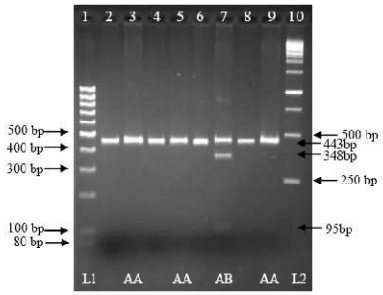

Fig. 6 Gel electrophoresis pattern obtained before and after digestion of amplified kappa-CSN fragment on original samples (gDNA); L1: 100bp ladder; L2: 1kb ladder.

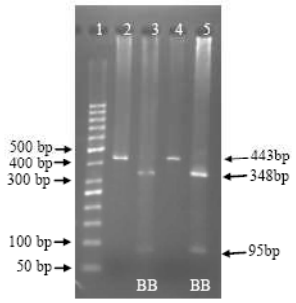

Fig. 7 Gel electrophoresis pattern obtained before and after digestion of amplified kappa-CSN fragment on ssI-PEP products of 2-cell samples

ADO, which is resulting from the failure of PCR to amplify one of the two alleles present in one cell; is unique to PCR of minute quantities of DNA [17].The observed mismatch is unlikely to be due to the structure of the optimized ssI-PEP procedure, because the occurrence of ADO has been reported by other researchers who utilized original I-PEP protocol [1], [14], [15], [18], [19]. This flaw has been even reported with MDA, which is an isothermal non-PCR-based procedure. It seems that ADO is a random and independent of the target fragment size and is most likely related to the inherent problems with single cell amplification [12], [20]. Moreover, there is a tendency toward increased complete locus and/or allele drop-out when the amount of starting template is less than 1ng [15]; and it disappears when increasing the starting cell count by 10-20 [12], [20]. However, in spite of the probability for ADO in specific analyses using WGA product, it is important that the average rate of ADO is still less than that for routine single cell PCRs using gDNA [17].

In conclusion, we propose that the efficiency and sensitivity of the optimized short and simple procedure for whole genome amplification in this study is the same as other WGA techniques, therefore this procedure is applicable for whole genome amplification of few bovine cells in PGD programs.

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