doi: 10.17221/29/2016-CJAS

Repeats As Global DNA Methylation Marker in Bovine Preimplantation Embryos

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ABSTRACT

Li W., Van Soom A., Peelman L. (2017): **Repeats as global DNA methylation marker in bovine preimplantation embryos.** Czech J. Anim. Sci., 62, 43–50.

DNA methylation undergoes dynamic changes and is a crucial part of the epigenetic regulation during mammalian early development. To determine the DNA methylation levels in bovine embryos, we applied a bisulfite sequencing based method aimed at repetitive sequences including three retrotransposons (*L1_BT*, *BovB*, and *ERV1-1-I_BT*) and *Satellite I*. A more accurate estimate of the global DNA methylation level compared to previous methods using only one repeat sequence, like *Alu*, could be made by calculation of the weighted arithmetic mean of multiple repetitive sequences, considering the copy number of each repetitive sequence. *Satellite I* and *L1_BT* showed significant methylation reduction at the blastocyst stage, while *BovB* and *ERV1-1-I_BT* showed no difference. The mean methylation level of the repetitive sequences during preimplantation development was the lowest at the blastocyst stage. No methylation difference was found between embryos cultured in 5% and 20% O_2. Because mutations of CpGs negatively influence the calculation accuracy, we checked the mutation rate of the sequenced CpG sites. *Satellite I* and *L1_BT* showed a relatively low mutation rate (1.92 and 3.72% respectively) while that of *ERV1-1-I_BT* and *BovB* was higher (11.95 and 24% respectively). Therefore we suggest using a combination of repeats with low mutation rate, taking into account the proportion of each sequence, as a relatively quick marker for the global DNA methylation status of preimplantation stages and possibly also for other cell types.

Keywords: epigenetics; retrotransposon; bisulfite sequencing

Preimplantation development from separate parental germ cells to a fertilized zygote and further to a blastocyst is a complex process including cell proliferation and differentiation. Epigenetic mechanisms play a crucial role here, allowing for activation of some genes and silencing of others. Among all the epigenetic mechanisms, DNA methylation is best known. It is associated with key processes in embryo development, including genomic imprinting, X-chromosome inactivation, and repression of repetitive elements to maintain the genome stability (Messerschmidt et al. 2014).

Bovine embryos can be successfully produced *in vitro* by various procedures. However, compared to *in vivo* embryos, *in vitro* produced and cloned embryos still show inferior quality and are associated with more losses during pregnancy (Farin et al. 2006). Furthermore, calves derived from *in vitro* produced embryos can be affected by the large offspring syndrome (LOS), and an altered methylation

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pattern that disturbs the gene expression during preimplantation is suggested as the primary cause for LOS (Young et al. 2001). The DNA methylation pattern is also suggested to be predictive of embryo quality during IVF (Aston et al. 2015). It is proposed that incomplete nuclear reprogramming in cloned embryos, which contributes to a low developmental success, is caused by failure of DNA demethylation (Bourc'His et al. 2001).

A common method used to study DNA methylation of preimplantation embryos is 5-methylcytosine (5-mC) immunofluorescence staining (Dean et al. 2001; Hou et al. 2007; Dobbs et al. 2013). However, conflicting results were found among these studies, for example as to whether methylation is more intensive in inner cell mass (ICM) or trophectoderm (TE) cells in IVF bovine blastocysts, and the staining result can be affected by the protocol used (Li and O'Neill 2012). Therefore, another simple and direct method is needed for determining the global DNA methylation in preimplantation embryos.

Bisulfite sequencing is another approach to evaluate DNA methylation at CpG dinucleotides, mostly applied for specific loci. The bisulfite (HSO_3^-) treatment converts cytosine residues to uracil, but leaves 5-methylcytosine residues unaffected (Frommer et al. 1992). By comparing the sequences the methylation pattern can be determined. More recently, new methods based on bisulfite conversion and whole genome sequencing have been developed (Guo et al. 2014; Salilew-Wondim et al. 2015). These platforms can determine the methylation status of thousands of genes at CpG site-level, but are still very expensive and require intensive bioinformatics (data analysis) expertise, which makes it impractical when a large number of treatments or samples are studied. Therefore, a genomic marker that is cheap, relatively easy to type, and representative for the global DNA methylation status is wanted.

Satellite DNA sequences have been used for this purpose, due to the presence of high order repeats that are from several hundred to several thousand bp in length (Sawai et al. 2011; Couldrey and Wells 2013). Besides satellite DNA, most of the DNA methylation in mammalian genomes is found in retrotransposons (Schulz et al. 2006). Retrotransposons, which account for almost half of the genome (44% of bovine genome (Adelson et al. 2009) and 45% of human genome (Lander et al. 2001)), were previously suggested as biomarker for global methylation status (Klose and Bird 2006). Recent research on epigenetics of mammalian preimplantation embryos found that transposable elements show a similar dynamic trend towards global methylation (Guo et al. 2014). Among all types of retrotransposons, L1 is mostly studied and has been used for indicating methylation changes in cancer cells (Hsiung et al. 2007), and a recent study of global methylation found a good correlation between L1 methylation and total amount of 5-methylcytosine measured by liquid chromatography mass spectrometry in murine cells and tissues (Newman et al. 2012).

In this paper, we selected three bovine retrotransposons with complete internal promoter sequence (*L1_BT*, *BovB*, and *ERV1-1-I_BT*), together with *Satellite I* DNA to be tested as marker for global DNA methylation estimation in bovine preimplantation embryo development. Since *in vitro* culture conditions alter DNA methylation (Salilew-Wondim et al. 2015) and different methylation patterns under oxygen stress (20% O_2) compared to normal (5% O_2) were found in a previous study by 5-methylcytosine immunofluorescence staining (Li et al. 2014), we included this in the study.

MATERIAL AND METHODS

Materials. Unless stated otherwise, all chemicals, reagents, and media were obtained from Sigma-Aldrich (USA) and Life Technologies (USA).

Primer design. Three autonomous retrotransposons were used for the methylation analysis (*L1_BT*, *BovB*, and *ERV1-1-I_BT*). First, retrotransposon sequences were retrieved from Repbase (Jurka et al. 2005). The amplicons were targeted to the internal promoter regions upstream of the open reading frame (ORF) from each retrotransposon sequence. In order to amplify as many as possible repeats to represent the whole genome, we used NCBI blast bovine genome (Btau_4.2) and MultiAlin (Hemberger 2007) to find the consensus sequence of each promoter, and then designed BSP primers based on the consensus sequences with BiSearch software (Aranyi et al. 2006). Primers are listed with predicted amplicon number in Table 1.

IVF embryo production and sample collection. *In vitro* bovine embryos were produced in serum-free media as previously reported (Li et al. 2014).

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The control group and high oxygen tension group were incubated at 38.5°C under 5% CO_2, 5% O_2 and 90% N_2, and 5% CO_2 in air (20% O_2) separately. Embryos were collected at specific time points (2–4-cell at 36–40 h post-insemination (h.p.i.), 8-cell at 64 h.p.i., expanded blastocyst at 8 days post-insemination).

Embryos were washed three times with phosphate-buffered saline (PBS), and then frozen in 3 pools of embryos for each stage (75 for 2–4-cell, 25 for 8-cell, and 5 for blastocyst) at –80°C until use.

DNA extraction and bisulfite conversion. Embryo DNA extraction and bisulfite conversion were performed with EZ DNA Methylation Direct™ Kit (Zymo Research, USA), according to the manufacturer's instructions with minor changes. Instead of incubating 20 min, we applied 3 h of incubation in M-Digestion Buffer and Proteinase K for embryo lysis and genomic DNA release, and the bisulfite converted DNA was eluted in 20 µl of water. Blood samples were taken from healthy cows, and genomic DNA was released with Proteinase K from 200 µl of blood. 20 µl of unpurified DNA solution was taken for bisulfite conversion directly with EZ Methylation-Gold™ Kit (Zymo Research), according to the manufacturer's instructions, and eluted in 20 µl of water. All converted DNA samples were checked by PCR with *GAPDH* primers (F: TTCAACGGCACAGTCAAGG; R: ACATACTCAGCACCAGCATCAC) to check for conversion completion. These primers amplify genomic DNA, but not converted DNA, so samples without a PCR amplicon, which represent samples without unconverted genomic DNA, were used for further methylation study.

Amplifying, cloning, and sequencing. After bisulfite treatment, retrotransposons and satellite DNA were amplified by PCR. To amplify the part of the repeat region, we used the following PCR mixture: 0.5 U FastStart™ Taq DNA Polymerase and 1 µl 10X reaction buffer (Roche, Switzerland), 200 µM deoxyribonucleotide triphosphates (dNTPs) (Bioline Reagents Ltd., UK), 500 nM of each primer (IDT, USA), and 2 µl of bisulfite converted DNA. The PCR program consisted of initial denaturation at 95°C for 5 min, followed by 30 cycles of 15 s at 95°C, 15 s at specific annealing temperature (Table 1), and 30 s at 72°C, and a final 10 min elongation at 72°C. The PCR products were verified by electrophoresis (2 µl out of 10 µl).

For each retrotransposon or satellite DNA, PCR products for three replicate samples were pooled, electrophoresed, purified (GENECLEAN II Kit; MP Biomedicals, USA), cloned in plasmid pCR2.1, and transformed into DH5α competent cells (Invitrogen, USA). Selected colonies were verified and amplified by PCR with universal and reversal primers (U: CGACGTTGTAAAACGACGGCCAG; R: CACAGGAAACAGCTATGACCATGATTACG), and those with a bright band on gel were sequenced.

Methylation analysis. Sequence analyses and statistical comparisons (nonparametric two-tailed Mann–Whitney test) were performed using the QUMA web service (quma.cdb.riken.jp). "Strict CpG site check of bisulfite sequence" was selected for repetitive sequence analysis. In this case, the

Table 1. Characteristics of the bisulfite primers used in the methylation analysis

Repetitive sequence name	Amplicon numbers[2]	Sequence 5' – 3'	Amplicon length (bp)	CpG numbers	Annealing temperature (°C)
Satellite I[1]	859	F: TTGGTTTTTAGGTTATGTAGGAG R: AATACACCAAACCCAATAAAAT	379	25	55
L1_BT	303	F: TAATTAAAATTTTTTGGGGGTTTG R: TAACCCTAAACTACATACACCTCCC	254	14	55
BovB	467	F: AGGAGGGTTTAGAGGAGTTATTTTA R: TCTATAAATATAATTTCAATATATTTACCC	210	6	58
ERV1-1-I_BT	44	F: TTTTTGGTATATTTTTATTTTATTT R: AATTATTAACTCCCATCTATAAAAA	214	11	55

[1]Wroclawska et al. 2010

[2]by BiSearch (Aranyi et al. 2006)

doi: 10.17221/29/2016-CJAS

divergence of retrotransposons is considered and a more corrected result can be obtained (Kumaki et al. 2008). Compared to regular genes, retrotransposons have a higher rate of mutation, so sequences that passed 90% bisulfite conversion were included in the analysis (normally 95% for genes). A *P*-value threshold of ≤ 0.05 was chosen to identify significant differences in the variance between groups.

Since different repetitive sequences have their own amplified number in the BS-PCR, we propose a method by using more than one retrotransposon as global methylation marker considering the proportion of each repetitive sequence by calculating the weighted arithmetic mean of the multiple repetitive sequences used, according to the following formula.

$$\bar{x} = \frac{\sum_{i=1}^{n} w_i x_i}{\sum_{i=1}^{n} w_i}$$

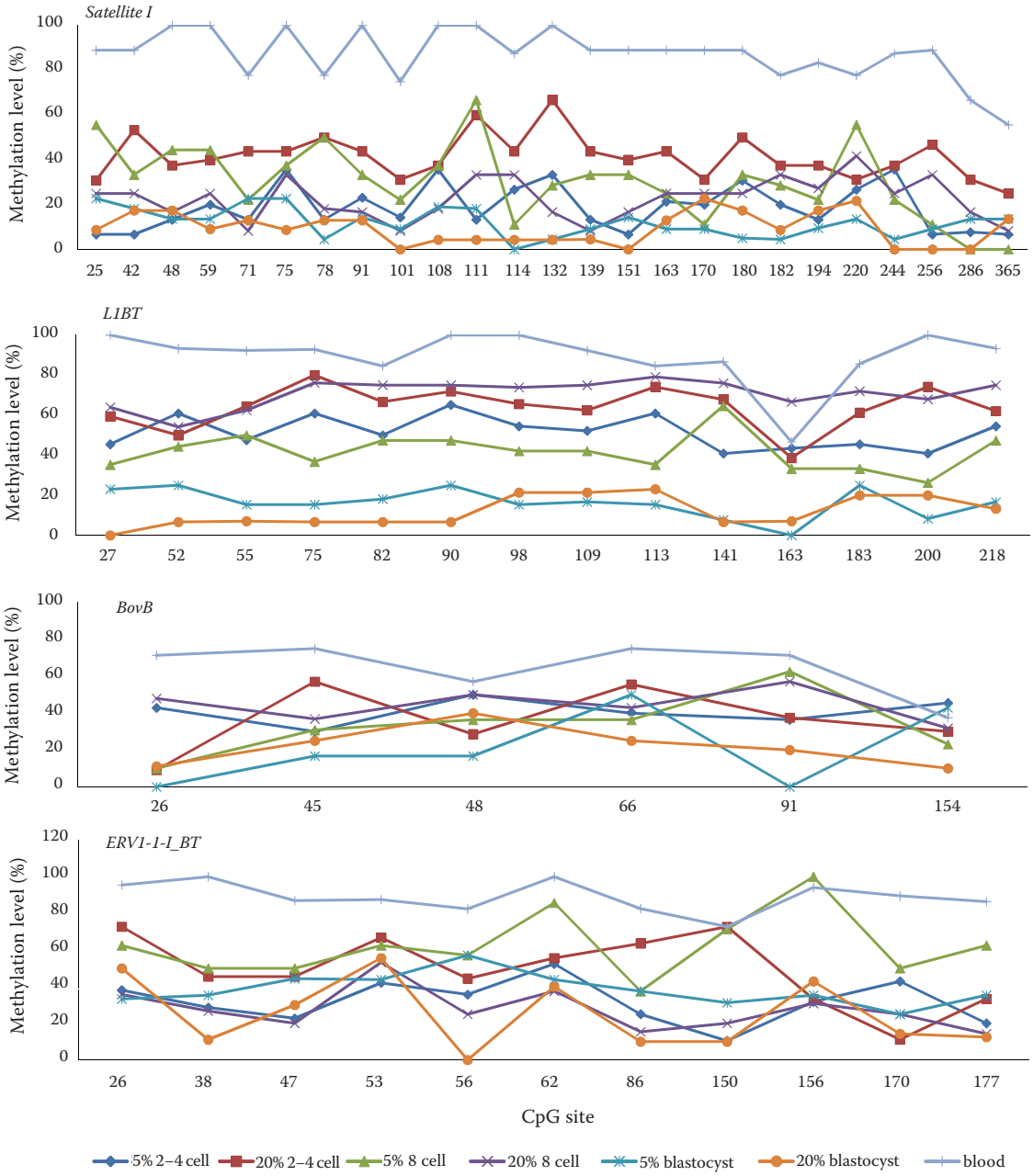

Figure 1. DNA methylation level of each CpG site on the repetitive sequences of embryos from each stage and treatment
X-axis shows the CpG sites on the amplified repetitive sequences, Y-axis shows the methylation level of each CpG site

doi: 10.17221/29/2016-CJAS

where:

x = methylation ratio of each repetitive sequence calculated by QUMA

n = number of repetitive sequences

w = amplicon number of each repetitive sequence from BiSearch (Table 1)

RESULTS

Methylation changes during embryo development. The DNA methylation levels of the four repetitive sequences in embryos and blood samples (used as reference) are indicated in Figure 1 (methylation level of each CpG site) and Figure 2 (methylation level of each repetitive sequence). As expected, the promoter regions in all four repetitive sequences were significantly hypermethylated in blood samples (with *Satellite I* 87.1%, *L1_BT* 89.5%, *BovB* 63.4%, and *ERV1-1-I_BT* 88.6% of CpGs methylated) compared to the preimplantation embryos. The methylation level of different stages of embryo development in standard culture (5% O_2) varies. In *Satellite I* DNA and *L1_BT*, there is a sharp decreased methylation in blastocyst as compared to the 2–4- and 8-cell stages (*P*-value of 0.0072 and 0.0175, respectively); in *ERV1-1-I_BT*, an increased methylation was found in 8-cell stage (*P*-value of 0.0362); while no significant methylation difference was found in *BovB*.

Methylation changes under high oxygen tension. Oxygen stress (20% O_2) does not seem to influence the methylation level of the four repeat families studied with the exception of *L1_BT* which was more methylated in the 8-cell stage under 20% O_2 (70.8%) compared to 41.8% under 5% O_2, with *P*-value of 0.0203 (Figure 2B).

Retrotransposon mutation frequency. Since repetitive sequences are variable, CpG sites may be present in some copies of the repeat and not in others. To estimate the CpG mutation frequency in the four repeats studied, we took the consensus sequence of the repetitive elements as reference sequence (Supplementary Figures S1 and S2) and applied the "strict CpG site check" on QUMA. In that case, TG is calculated as unmethylated; CG as methylated, and others as site mutation. The mutation rate thus estimated was 1.92% in *Satellite I*, 3.72% in *L1_BT*, 24.03% in *BovB*, and 11.95% in *ERV1-1-I_BT*.

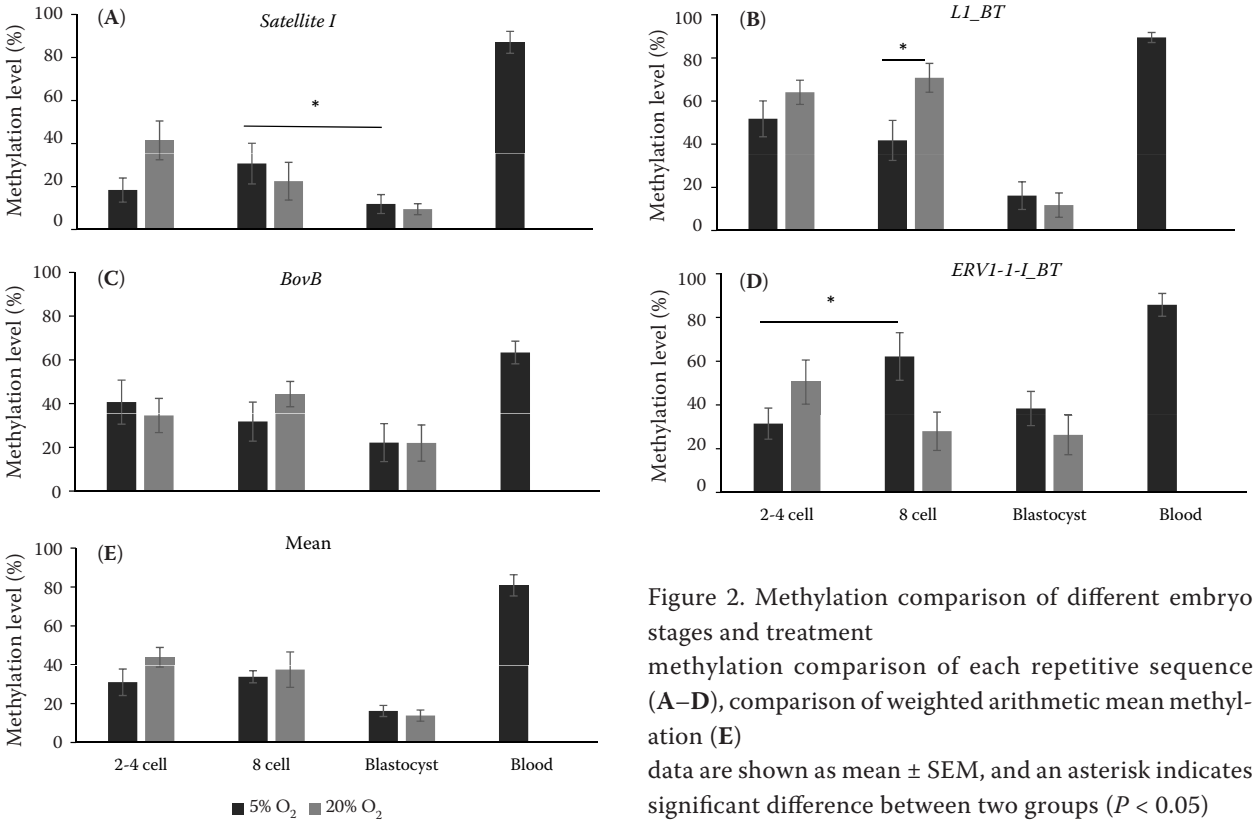

Figure 2. Methylation comparison of different embryo stages and treatment

methylation comparison of each repetitive sequence (**A**–**D**), comparison of weighted arithmetic mean methylation (**E**)

data are shown as mean ± SEM, and an asterisk indicates significant difference between two groups ($P < 0.05$)

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Global methylation estimation by combining the four repetitive sequences. The global methylation level was calculated by using the weighted arithmetic mean of the four repetitive sequences studied as shown in Figure 2E. No significant methylation difference was observed between embryos produced under 5% O_2 and 20% O_2 culture, but methylation at the blastocyst stage was much lower than that in earlier stages.

DISCUSSION

The alteration of DNA methylation patterns in preimplantation embryo development has been studied widely in different species (Bourc'His et al. 2001; Fulka et al. 2004). Global DNA demethylation and *de novo* methylation in this period has been shown to guide and restrict differentiation and prevent cell regression into an undifferentiated state, and on the other hand are also crucial to establish pluripotency (Messerschmidt et al. 2014). An accurate quantitative assay representative for global DNA methylation may not exist, but repetitive genomic sequences such as satellite DNA and L1 which account for almost 20% of the mammalian genome have been widely used as a means for estimating global methylation status in cancer research (Yang et al. 2004).

In the present study, we evaluated bisulfite sequencing of four repetitive sequences as a global DNA methylation marker during bovine early embryo development and used it to check if oxygen stress (20% O_2) has an influence on the global methylation level of preimplantation embryos. *Satellite I* and *L1_BT* showed the lowest methylation level at the blastocyst stage. This is consistent with *DNA cytosine-5-methyltransferase1* (*DNMT1*) RNA expression and the results of a recent report (Salilew-Wondim et al. 2015). We previously found *DNMT1* expression dropped markedly in blastocysts compared to earlier stages (4-cell embryo) (Li et al. 2014), implying hypomethylation in the blastocyst. However, the *DNMT1* expression was not corroborated by the DNA methylation level as measured by 5-mC immunofluorescence staining which indicated the highest level in the blastocyst stage (Li et al. 2014). In other species, conflicting results were found as well. In human embryos, 5-cytosine immunofluorescence staining showed an increased methylation in blastocysts compared to morula (Fulka et al. 2004), while Guo et al. (2014) found, by using a bisulfite sequencing based method – reduced representation bisulphite sequencing (RRBS), the lowest DNA methylation level in blastocysts. The same conflicting results were found also in mouse embryos, with high methylation levels compared to previous stages found in blastocysts by staining (Dean et al. 2001), whereas RRBS showed the lowest methylation level in mouse morula/blastocyst stages (Smith et al. 2012). These conflicting results were not only found between these two analytical methods, but also between studies using the same staining method. There is disagreement in whether methylation is higher in ICM or TE of blastocysts (Dean et al. 2001; Hou et al. 2007; Dobbs et al. 2013; Li et al. 2014), and differences in staining protocols may be responsible for the conflicting results.

There are several possible explanations for the different results obtained with 5-mC staining. First of all, changes in the staining protocol may lead to different results. Li and O'Neill (2012) used a step of trypsin digestion in methylation staining and a different result of paternal demethylation during mouse zygote maturation was found. Besides the staining method itself, the lack of standard quantification for staining may contribute to different results as well. The ratio of 5-mC to a DNA counter stain is often used for methylation level estimation. The most common dyes for DNA counter staining are Hoechst, DAPI, propidium iodide (PI), and ethidium homodimer-2 (EthD-2) which only bind to double-stranded DNA, or weakly bind to single-stranded DNA and RNA as well. Therefore, this widely used ratio presents actually single-stranded methylated DNA/total double-stranded DNA, which can be easily influenced by the denaturation of DNA. Furthermore, the fluorescence intensity observed may be affected by cell size or shape and even the placement orientations under the microscope. The compact composition of morula and ICM in blastocysts also makes the staining and methylation quantification more difficult.

Bisulfite sequencing of repetitive sequences, on the other hand, has its limitations as well and we tried to improve on that. First of all, the high mutation rate of repetitive sequences makes the number of potential targets for DNA methylation variable between the individual members of a repeat family. This is the reason we did not

doi: 10.17221/29/2016-CJAS

include SINEs in the study, since they are super mutated. For example, almost two-thirds of the CpG methylation sites in *Alu* elements (the most widespread SINE family in the human genome) are mutated (Yang et al. 2004). In the present study, we calculated the mutation rate of CpG sites of the four repetitive sequences studied. *Satellite I* showed the lowest mutation rate (1.92%), while *BovB* had the highest mutation rate (24.03%) making it less suitable as global methylation marker when used individually.

Another factor that affects the methylation result is primer design, which can influence the number of amplified repetitive sequence directly. For example, the *Alu* bisulfite primers used in a recent study could amplify only 16 different copies, with length differing from 46 up to 1017 bp (Lisanti et al. 2013), making it dubious as marker for global methylation status. In the present study, we tried to amplify as many as possible copies of the repeat families to represent the whole genome. All primers were designed based on the consensus sequence of the promoters and the amplified number (listed in Table 1) could be checked by BiSearch. To improve the method even more we calculated the weighted arithmetic mean of the four repetitive sequences studied and used it for calculation of the global methylation status.

CONCLUSION

In conclusion, this study is the first report using *L1_BT*, *BovB*, *ERV1-1-I_BT*, and *Satellite I* DNA as global methylation marker in bovine early embryo development. The mean methylation of the repeats showed the same tendency as observed in a recent DNA methylation study (Salilew-Wondim et al. 2015), with blastocysts showing the lowest methylation level. We also compared DNA methylation in embryos cultured under normal (5% O_2) with high oxygen tension (stress group, 20% O_2), and found only significant hypermethylation in *L1_BT* at 8-cell under stress. Although the accuracy and sensitivity of the method used needs to be tested in more independent studies, we propose using the weighted arithmetic mean of several repetitive sequences as global methylation marker. Especially the repeats with (relatively) low mutation rate and more copies targeted during PCR, like *Satellite I* DNA and *L1_BT*, are promising.

Acknowledgement. The authors would like to thank Petra Van Damme and Isabel Lemahieu for their excellent technical assistance with *in vitro* culture of embryos. We also thank Linda Impe for the great help with colony PCR and sequencing.

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Received: 2016–03–31
Accepted after corrections: 2016–10–03