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# THE BISULPHITE-SEQUENCING PCR FOR THE BOVINE *GSTP1*, *GSTA4*, AND *ACHE* GENE METHYLATION STUDY

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## ABSTRACT

**Modifying cytosine by adding a methyl group in CpG dinucleotides within DNA sequence has several functions, but acts primarily as an important regulatory mechanism of mammalian gene expression. This consequently affects the phenotypic characteristics of the organism. The methylation profiles can be altered as a consequence of various environmental influences, age, disease, or diet, and can serve as biomarkers of important production and health traits. In this regard, the methylation profile analysis techniques are important. In the present study, we proposed and tested specific oligonucleotide primers for the bisulphite-sequencing polymerase chain reaction (BSP) analysis of the bovine *GSTP1*, *GSTA4*, and *AChE* genes, to optimize this approach for future studies of the methylation status of these genes. BSP can be used, for example, to study the effect of different pesticides and their combinations on the methylation of these and other bovine genes.**

**Key words: acetylcholinesterase; bisulphite sequencing; bovines; DNA methylation; glutathione-S-transferase A4; glutathione-S-transferase P1; primer**

## INTRODUCTION

DNA methylation involves adding a methyl group to the DNA molecule, which can influence gene expression and, consequently, relevant phenotypic traits. This epigenetic modification of DNA participates in different physiological processes, such as reproduction, fertility, epigenetic inheritance, health, and disease [7]. In mammals, the most frequent DNA methylation mark is 5-methylcytosine (5mC) present mainly in the CpG dinucleotide context; however, it also appears in the CHG and CHH contexts, where H is either A, C or T [3]. The condensed areas of CpG dinucleotides are known as CpG islands (CGIs) which are mostly located at promoters of housekeeping genes or genes involved in development [9]. The hypermethylation of CGIs may affect gene regulation by inhibiting gene transcription; on the other hand, hypermethylation of

gene bodies can lead to stimulation of gene expression [4]. DNA methylation profiles of organisms are not stable but are affected by environmental factors, age, diet, sex or various diseases. The corresponding methylation patterns can serve as biomarkers of important production and health traits, therefore techniques of analysing the methylation status of the whole genome or of individual genes are important. Multiple methods used to study DNA methylation profiles include methylation-specific polymerase chain reaction (MSP), methylated DNA immunoprecipitation, and bisulphite sequencing [7]. The bisulphite sequencing techniques use a sodium bisulphite treatment to convert non-methylated C to T while 5mC is resistant to the modification and remains unchanged. Before sequencing, the bisulphite-treated DNA undergoes PCR amplification with an uracil-tolerant polymerase to provide sufficient template for analysis, whereby U changes to T. After traditional sequencing (Sanger or Illumina short-read), DNA methylation can be read directly through comparison to a reference or untreated sequence [3]. Bisulphite sequencing of promoter stretches, or first exon regions, amplified using specific primers designed by different online available software, allows methylation analysis of individual genes.

Glutathione-S-transferases (GSTs) are a group of multifunctional enzymes abundant in both, prokaryotes and eukaryotes. In eukaryotes, several groups of GSTs are found, according to the location in the cell; namely cytosolic, mitochondrial, and microsomal [1]. GSTs have multiple biological functions, including the main role, which is the detoxification of the cells from several toxic molecules and their protection against oxidative stress. Besides the enzymatic activity, GSTs exhibit also non-enzymatic functions such as modulation of signal transduction in cell survival and apoptotic pathways. Pathological expression of GSTs can induce some diseases, such as oncological or neurodegenerative ones. In humans, cytosolic GSTs comprise several classes, according to their chemical, structural and physical properties: alpha (GSTA), zeta (GSTZ), theta (GSTT), mu (GSTM), pi (GSTP), sigma (GSTS), and omega (GSTO).

Acetylcholinesterase (AChE) is present in many organisms where it regulates the levels of the neurotransmitter acetylcholine via its hydrolysis, playing an important role in cholinergic synapsis of the central and peripheral nervous system [2]. The protein can also participate in inflammation, apoptosis, and oxidative stress [13].

In the present study, we proposed and tested specific oligonucleotide primers for the bisulphite-sequencing PCR (BSP) analysis of the bovine *GSTP1, GSTA4*, and *AChE* genes in order to optimise this approach for the qualitative determination of the methylation status of these genes. In the future, the BSP will allow the analysis of the methylation status of these genes for various purposes, for example, to study the impact of various pesticides and their mixtures.

## MATERIAL AND METHODS

### The template DNA and its bisulphite modification

Commercially available bovine genomic DNA (Novagen-Millipore, EMD Millipore Corp., Burlington, MA, USA) modified with bisulphite by MethylEdge™ Bisulfite Conversion System (Promega, Madison, WI, USA), was used as a template in the amplification reactions.

### Designing the primers and BSP

BSP primers for amplification of *GSTP1*, *GSTA4*, and *AChE* genes were designed using MethPrimer 1.0 software available online (https://www.urogene.org/cgi-bin/methprimer/methprimer.cgi), accessed on 25th October 2023 and 8th February 2024 (Table 1). The concentrations and amounts of individual reagents in the 25 µl reaction mixture were as follows: 1xGoTaq® G2 Hot Start Polymerase reaction buffer (Promega), 1.5 and 2.5 mM $MgCl_2$ (Promega) for the *GSTP1* and *AChE* genes or the *GSTA4* gene, 0.2 mM dNTPs (Promega), 0.25 µM of both the forward and reverse primers (Microsynth AG, Balgach, Switzerland), 0.125 µl of GoTaq® G2 Hot Start Polymerase (5 $U.\mu l^{-1}$, Promega), nuclease-free water, and about 10–20 ng of the bisulphite-treated bovine DNA. The amplification conditions were optimized as follows: I/95 °C, 2 min; II/35 cycles: 95 °C, 40 sec; 54, 50, and 52 °C (for the *GSTP1*, *GSTA4*, and *AChE* gene, respectively), 30 sec; 72 °C, 1 min; III/72 °C, 5 min. The amplifications were run in the thermocycler Biometra (Analytik Jena). In each analysis, the NTC (non-template control) with 1 µl of water instead of template DNA was included. The amplification products were checked in the 1.5 % agarose gel stained with GelRed® Nucleic Acid Stain (Biotium) and purified by Monarch® DNA Gel Extraction Kit (New England Biolabs, Ipswich, Massachusetts, USA).

**Table 1. Properties of primers designed by MethPrimer 1.0 software available online (https://www.urogene.org/cgi-bin/methprimer/methprimer.cgi), accessed on October 25, 2023 and February 8, 2024, for BSP analysis of methylation status of the *GSTP1*, *GSTA4*, and *AChE* gene**

| Primer name | Primer sequence | Primer length (bp) | PCR product size (bp) |
|---|---|---|---|
| **GSTP1-BS1-F** | 5′- GTTTTGAGGATGTTGTGTTTAGGTG - 3′ | 25 | 237 |
| **GSTP1-BS1-R** | 5′- TATATTTAAAATCCTACTCCACCC - 3′ | 24 | |
| **GSTA4-BS1-F** | 5′- GAGTTTGTTTGGAGGTTTTAGTAGG - 3′ | 25 | 293 |
| **GSTA4-BS1-R** | 5′- TCCTTAAAAACAAAAATTTCTTATAATTTA - 3′ | 30 | |
| **AChE-BS2-F** | 5′- GTGGTTAGGGATGTTTATTTATT - 3′ | 23 | 285 |
| **AChE-BS2-R** | 5′- AACCCAAACCCAAAAACCCC - 3′ | 20 | |

GeneRuler 100 bp DNA ladder (Thermo Fisher Scientific Baltics UAB, Vilnius, Lithuania) was used as the molecular weight marker. Sequencing of PCR products obtained with the BSP primers was carried out commercially (Laboratory of Biomedical Microbiology and Immunology, University of Veterinary Medicine and Pharmacy in Košice, Slovak Republic and Eurofins Genomics Germany GmbH, Ebersberg, Germany).

**Sequence alignment and evaluation of the C methylation status in the CpG dinucleotides**

The sequences of amplification products obtained with the *GSTP1*, *GSTA4*, and *AChE* BSP primers were compared with the gene sequences in the online database (ensembl.org;
*GSTP1*: **ENSBTAG00000003548** 29:45409548-45412284:1;
*GSTA4*: **ENSBTAG00000004288** 23:25209831-25224178:-1;
*AChE*: **ENSBTAG00000001139** 25:35763126-35768952:1)

using the online available Clustal Omega software (https://www.ebi.ac.uk/jdispatcher/msa/clustalo), accessed on 27th and 28th May, and 21st June 2024. Assessment of the methylation status of the studied genes consisted of evaluating CpG dinucleotides in stretches defined by BSP primers amplified with the bisulphite-modified template DNA in terms of C to T change. If altered nucleotide was identified, the corresponding C in the appropriate CpG dinucleotide was considered unmethylated. Otherwise, the presence of the methylated cytosine will not cause a C to T change but the C nucleotide is preserved.

## RESULTS

Using the designed BSP primers for individual genes (Table 1) in PCR reactions, fragment of the *GSTP1* (237 bp), *GSTA4* (293 bp), and *AChE* gene (285 bp) were amplified and were detected by electrophoretic analysis (Fig. 1). After purification, each amplification product was sequenced and the sequences were compared with the sequences of the corresponding gene in the online database. Based on the approach outlined in the Material and Methods section, we evaluated the methylation status of C in these alignments in the following numbers of CpG dinucleotides: 17 in the case of the *GSTP1* gene, 19 in the case of the *GSTA4* gene, and 22 in the case of the *AChE* gene. Figure 2 presents the sequence alignment of the 285 bp fragment of the *AChE* gene. We found that in all CpG dinucleotides for each gene that we evaluated, C changed to T, indicating that C was not methylated in those CpG dinucleotides of standard bovine genomic DNA.

## DISCUSSION

In the present study, we designed and tested primers for bisulphite-sequencing PCR of the bovine *GSTP1*, *GSTA4*, and *AChE* genes, to optimize this method for assessing their methylation status. Since the exact location of the promoters of the studied genes is unknown, but the location of the 5′-UTR region and the individual exons of these genes are annotated in the ENSEMBL database, we

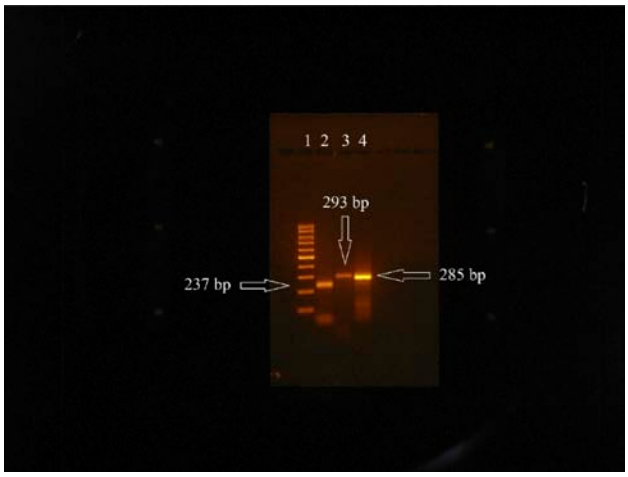


**Fig. 1. The electrophoretic analysis of amplification products generated by the BSP primers for the *GSTP1*, *GSTA4*, and *AChE* genes. Lane 1 – GeneRuler 100 bp DNA ladder (Thermo Fisher Scientific Baltics UAB, Vilnius, Lithuania); Lane 2 – 237 bp band of the *GSTP1* gene; Lane 3 – 293 bp band of the *GSTA4* gene; Lane 4 – 285 bp band of the *AChE* gene**


**Fig. 2. The alignment of the sequence of the gene fragment amplified with the BSP-AChE primers (test 2) with the reference sequence of this gene in the ENSEMBL database (test 1) obtained by the Clustal Omega software (https://www.ebi.ac.uk/jdispatcher/msa/clustalo), accessed on 21st June, 2024. CpG dinucleotides evaluated in terms of C to T change in the studied region are highlighted in red and stars indicate consent nucleotides in both compared sequences.**

targeted our designed primers to amplify stretches probably in the promoter region, or first exons, of these genes. Our results indicate that each cytosine in the CpG dinucleotides present in the analysed regions of all genes we studied was converted to thymine what indicates that none of the cytosine was methylated. These results correspond with the statement that while most methylated CpGs remain methylated throughout development, CpGs present in CG-dense regions – CGIs, are hypomethylated [8, 11].

In our previous study, we used MSP to study methylation of the *GSTP1*, *GSTA4*, and *AChE* genes in bovine lymphocytes cultured *in vitro* with the insecticide Mospilan 20SP alone and in combination with fungicides Orius 25EW and miconazole [5, 6]. MSP is a sensitive technique, that permits the detection of methylation at high throughput, but this is limited to the primer binding sites [12]. Coupling bisulphite conversion with DNA sequence analysis enables the qualitative and quantitative detection of 5mC at a single base-pair resolution. Following bisulphite conversion, DNA is analysed with next-generation transcriptomics to validate the genome-wide DNA methylation profile, and the regional methylation status established by Sanger sequencing [10]. Our results indicate that the mentioned combination of bisulphite conversion with sequencing analysis allowed us to analyse the methylation status of C in CpG dinucleotides within stretches defined by specific primers designed in the region around the 5′-UTR sequence of the bovine *GSTP1*, *GSTA4* and *AChE* genes. Moreover, we could analyse more CpG dinucleotides with this approach than with MSP. In the future, it will be possible to use BSP to evaluate qualitatively the methylation status of any region defined by the designed primers of any bovine gene for many purposes, including assessment of methylation changes under the influence of different pesticides and their combinations.

## CONCLUSIONS

The bisulphite-sequencing PCR using primers we designed is suitable for studying the methylation status of bovine *GSTP1*, *GSTA4* and *AChE* genes.

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