Original Article

Study of the Methylation of Bovine *GSTP1* Gene under the Influence of Pesticide Mospilan 20SP Alone and in Combination with Pesticide Orius 25EW

(DNA methylation / *GSTP1* / acetamiprid / tebuconazole / *Bos taurus* / methylation-specific PCR)

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Abstract. DNA methylation, one of the most studied epigenetic mechanisms, when present in the promoter region of genes, causes inhibition of gene expression, and conversely, hypomethylation of these regions enables gene expression. DNA methylation is susceptible to nutritional and environmental influences, and undesirable alterations in methylation patterns manifested in changes in the expression of relevant genes can lead to pathological consequences. In the present work, we studied the methylation status of the bovine *GSTP1* gene under the influence of pesticide Mospilan 20SP alone and in combination with pesticide Orius 25EW in *in vitro* proliferating bovine lymphocytes. We employed methylation-specific PCR, and when studying the effect of pesticide combinations, we also used its real-time version followed by a melting procedure. Our results showed that Mospilan 20SP alone at 5, 25, 50, and 100 µg.ml^{-1} and 5, 10, 25, and 50 µg.ml^{-1} for the last 4 and 24 hours of culture with *in vitro* proliferating bovine lymphocytes, respectively, did not induce methylation of the bovine *GSTP1* gene. The same results were revealed when studying the effect of the combination of the pesticides added to the lymphocyte cultures for the last 24 hours of cultivation in the following amounts: 1.25, 2.5, 5, 10, and 25 µg.ml^{-1} of Mospilan 20SP and 1.5, 3, 6, 15, and 30 µg.ml^{-1} of Orius 25EW. We have also revealed that the less laborious real-time MSP followed by a melting procedure may replace MSP for studying the methylation status of the *GSTP1* gene.

Introduction

Epigenetic regulation involves changes in gene expression that are not due to alterations in the DNA sequences themselves (Pechalrieu et al., 2017). Instead, gene expression is modulated by DNA methylation, histone acetylation, the action of non-coding RNAs or Polycomb/Thritorax group proteins. DNA methylation within the eukaryotic genome is present in cytosines that are mainly part of CpG dinucleotides, and while most methylated CpGs remain methylated throughout development, CpGs present in so-called CpG islands (CG-dense regions) are hypomethylated (Smith and Meissner, 2013; Lyko, 2018). DNA methylation of regulatory sequences, such as promoters and enhancers, results in the repression of downstream genes or transposons; however, methylation of gene bodies can cause gene activation (Neri et al., 2017; Greenberg and Bourc'his, 2019). DNA methylation is not stable, and the methylation status of the genome is the result of the interaction of genetic, epigenetic, and environmental factors (Dhar et al., 2021). Techniques for methylation analysis should be characterized by accuracy, sensitivity, speed, simplicity, and optimal financial cost. Based on DNA pretreatment, they can be divided into the following groups: i) restriction enzyme digestion-based techniques, ii) bisulphite-based techniques, and iii) affinity enrichment-based techniques (Halabian et al., 2021). One of the most widely used bisulphite tech-

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Abbreviations: GST – glutathione S-transferase, MSP – methylation-specific PCR, NTS – non-template sample, PCR – polymerase chain reaction.

niques is methylation-specific PCR (MSP), which, after conversion of unmethylated cytosines to uracils and retaining methylated cytosines due to bisulphite treatment, uses selective primers for amplification of methylated and unmethylated DNA.

Pesticides are a chemically diverse group of substances used in agriculture to protect crops and control food production. So far, many environmental toxins, including pesticides, have been found to cause changes in the methylation status of the genome (Paul et al., 2018). Woźniak et al. (2020) observed changes in methylation patterns of *P21* and *TP53* genes in human peripheral blood mononuclear cells (PBMCs) exposed to glyphosate. Mahna et al. (2021) reviewed various studies in animal models and human samples pointing out that different types of pesticides (e.g., endocrine disruptors, herbicides and insecticides) may cause global methylation modifications of the genome or changes in methylation of specific genes. It was revealed that the exposure of F_0 rats to glyphosate induced differentially methylated regions (DMRs) in genes associated with pathologies such as prostate, ovarian and kidney diseases, as well as obesity or parturition abnormalities in F_1, F_2 and F_3 progeny (Kubsad et al., 2019). Bebane et al. (2019) found numerous genes with differential expression between bees exposed to neonicotinoid imidacloprid and control bees, but not differentially methylated cytosines (DMCs). Exposure of the whole oyster tissue to diuron was shown to decrease global DNA methylation and total methyltransferase activity (Akcha et al., 2020). Global DNA methylation levels were increased in the ovary of zebrafish treated with pesticide flutolanil, resulting in gonad endocrine disruption, decreased reproduction and developmental toxicity in F_1 progeny (Teng et al., 2020).

Glutathione S-transferases (GSTs) represent an important group of detoxification enzymes, and the *GSTP1* gene is one of the most studied members of the group. Increased methylation of the promoter region of the *GSTP1* gene may occur under certain unfavourable conditions, which would inhibit the normal gene expression and could lead to the reduction or loss of its detoxification and antioxidant activities. So far, increased promoter methylation of this gene and its pathological consequences have been mainly investigated in humans. Cui et al. (2020) reviewed the association of *GSTP1* gene expression with the development and treatment of various types of cancer and discussed the methylation of the gene in prostate, breast, and lung cancer as well as in hepatocellular carcinoma. It was found that acromegaly patients with the methylated *GSTP1* gene promoter showed a higher prevalence of diabetes mellitus and colonic polyps and were more resistant to treatment with somatostatin analogues (SSA) than patients with the unmethylated *GSTP1* gene promoter (Ferraù et al., 2019). Zhang et al. (2019) showed that along with others, the *GSTP1* gene promoter in urine samples of the population with high levels of arsenic in drinking water expressed higher susceptibility to methylation in individuals who carried the DNMT3B (SNP rs2424932) GA genotype. Hernández et al. (2018) identified differential hypermethylation in several genes, including *GSTP1*, in cortical pyramidal layers susceptible to late-onset Alzheimer's disease (LOAD) neurodegeneration.

The bovine *GSTP1* gene shows more than 85 % homology of amino acid sequence with humans (Hernando et al., 1992). This gene should normally be unmethylated and its increased methylation could harm the bovine organism. In the present work, we studied the methylation status of the bovine *GSTP1* gene under the influence of pesticide Mospilan 20SP alone and in combination with pesticide Orius 25EW in *in vitro* proliferating bovine lymphocytes. For this purpose, we used methylation-specific PCR (MSP), verifying the accuracy of the designed primers by sequencing PCR products and comparing them with the gene sequence in the online database before the actual analysis. To study the effect of the combination of the above pesticides, after its optimization, we used the real-time version of MSP followed by a melting procedure in addition to MSP to determine its suitability for studying the methylation status of the *GSTP1* gene.

Material and Methods

Blood collection and cultivation of lymphocytes with the pesticides

Collection of peripheral blood from two healthy bull donors (Slovak spotted cattle, 5–6 months, housed at the Clinic of Ruminants, University Veterinary Hospital, University of Veterinary Medicine and Pharmacy in Košice, Slovak Republic) and cultivation of lymphocytes with the pesticides was performed as has been previously described (Halušková et al., 2019). In an attempt to study the effect of MOSPILAN® 20SP (20.2 % acetamiprid CAS 135410-20-7; 2.4 % benzene sulphonic acid CAS 90194-45-9) alone, the following amounts dissolved in water were added to the lymphocyte cultures: 5, 25, 50, and 100 $\mu g.ml^{-1}$ and 5, 10, 25, and 50 $\mu g.ml^{-1}$ for the last 4 and 24 hours of cultivation, respectively. In an attempt to study the effect of pesticide combination, Mospilan® 20SP and Orius® 25EW (25 % tebuconazole CAS 107534-96-3; 61 % (60–70 %) N, N-dimethyldecanamide CAS 14433-76-2) diluted in dimethyl sulphoxide (DMSO) were added to the lymphocyte cultures for the last 24 h of cultivation at the amounts of 1.25, 2.5, 5, 10, 25 and 1.5, 3, 6, 15, 30 $\mu g.ml^{-1}$, respectively. Negative controls were prepared by adding water or DMSO instead of pesticide solutions.

Lymphocyte DNA isolation, its bisulphite modification and preparation of fully methylated bovine DNA

Isolation of DNA from lymphocytes and its modification with bisulphite was performed as previously mentioned (Halušková et al., 2019). Fully methylated bo-

vine DNA that served as the positive control in the MSP amplification reactions with primers for the methylated *GSTP1* gene was prepared in 50 µl reaction volume by adding the following reaction components: 5 µl of 10× concentrated SssI methyltransferase reaction buffer (New England Biolabs – NEB, Ipswich, MA); 5 µl of the 20× diluted stock solution of S-adenosylmethionine (SAM; NEB); approximately 1 µg of DNA (Novagen-Millipore, EMD Millipore Corp., Burlington, MA; 5 µl); 1 µl (4 U) of SssI methyltransferase (NEB), and 34 µl of sterile water. The mixture was incubated for 1 h at 37 °C and then directly precipitated with 0.8 volume of isopropanol, centrifuged, washed with 70 % ethanol, and dissolved in the elution buffer from the DNA isolation kit (Promega, Madison, WI). The appropriate amount of the methylated DNA was bisulphite-converted and analysed by MSP.

MSP, real-time MSP followed by a melting procedure and sequencing of PCR products

MSP including the designing of primers was performed as previously described (Halušková et al., 2019) except that the annealing temperature of 54 °C was used in amplification reactions with primers for the unmethylated *GSTP1* gene. Also, in addition to commercially available standard bovine DNA from Sigma-Aldrich (Saint Louis, MO), DNA from Novagen-Millipore was analysed by MSP, and in the electrophoretic analysis, 100 bp DNA ladders from SBD (Solis BioDyne, Tartu, Estonia) and SBS (SBS Genetech Co., Ltd., Beijing, China) were used as molecular weight standards.

The 20 µl real-time MSP reaction mixtures contained 10 µl of 2× concentrated Power SYBR® Green PCR Master Mix (Applied Biosystems by ThermoFisher Scientific, Waltham, MA), 1 µl (0.5 µmol.l^{-1}) of both forward and reverse primers, 2 µl (10–20 ng) of bisulphite-modified template DNA and 6 µl of sterile water. The real-time MSP amplifications were performed in LightCycler 480 (Roche) and the amplification conditions were as follows: I) 95 °C, 10 min, II) 40 cycles: 95 °C, 15 s; 54 °C and 60 °C (primers for the unmethylated *GSTP1* gene), and 55 °C and 61 °C (primers for the methylated *GSTP1* gene), 30 s; 72 °C, 30 s, III) melting procedure: 95 °C, 15 s; 60 °C, 1 min; 95 °C, 30 s. In the case of real-time MSP reactions with primers for the unmethylated *GSTP1* gene, when the annealing temperature of 60 °C was used, according to the producer's recommendations, step II), the 72 °C, 30 s sub-step was omitted. In each amplification reaction, the non-template sample (NTS) with added sterile water instead of template DNA was included.

Sequencing of PCR products obtained with primers for both unmethylated and methylated *GSTP1* gene in the classical MSP reactions was carried out commercially (Laboratory of Biomedical Microbiology and Immunology, University of Veterinary Medicine and Pharmacy in Košice, Slovak Republic).

Results

Verification of MSP primers

We intended to validate the MSP primers previously designed for both unmethylated and methylated bovine *GSTP1* gene by comparing the sequences of the MSP amplification products with the sequences of the stretches flanked by the primers in the online database. First, we sequenced the products of four amplification mixtures obtained with primers for the unmethylated *GSTP1* gene and with the template DNA isolated from lymphocytes of one of two animals cultured without pesticides. We used this DNA as a template in the above reactions because we assumed that under normal conditions, the *GSTP1* gene is unmethylated. Similarly, we sequenced the amplification products from two amplification mixtures obtained with primers for the methylated *GSTP1* gene and template DNA, which was a fully methylated commercial standard bovine DNA prepared by us. A comparison of the sequences of the amplification products with the original *GSTP1* gene sequences in the ENSEMBL database showed that they fully matched, demonstrating that the primers for the unmethylated and methylated bovine *GSTP1* gene were correctly designed. Figure 1 shows the alignment of the sequence of the PCR product obtained with primers for the methylated *GSTP1* gene and fully methylated standard bovine DNA as a template, with the original sequence of the gene in the ENSEMBL database.

MSP study of the bovine GSTP1 gene methylation in lymphocytes treated with Mospilan 20SP alone and in combination with Orius 25EW

MSP analysis with primers for the unmethylated and methylated *GSTP1* gene was performed in bisulphite-modified DNA samples isolated from lymphocytes of both animals cultivated without (control) and with Mospilan 20SP alone and in combination with Orius 25EW (experiment). The bisulphite-treated commercial standard bovine DNA (presumed unmethylated) and its fully methylated version were also analysed by MSP as positive controls in reactions with primers for the unmethylated and methylated *GSTP1* gene, respectively.

Amplification with primers for the unmethylated *GSTP1* gene should result in a band of 209 and that for the methylated gene in a band of 200 bp. Our study indicated that the expected 209 bp band was present in all DNA samples amplified with primers for the unmethylated *GSTP1* gene that were isolated from lymphocytes cultured without and with all amounts of pesticide Mospilan 20SP alone as well as in combination with pesticide Orius 25EW. The same 209 bp band was also present in the standard bovine DNA samples amplified with primers for the unmethylated *GSTP1* gene (Fig. 2A).

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           *  *********** ** *******  ******* ******     ******************************
GSTP1_Met  GTTCGGCGGACGTTTAGTAATCGGTTTCGGCGAGTTGCGGGTTTTTAGGGGATGGAGATTAAGCGGTGGGGCGGG
GSTP1_db   GCCCGGCGGACGTTCAGCAATCGGTCCCGGCGAGCTGCGGGCCCCCAGGGGATGGAGATTAAGCGGTGGGGCGGG

           *********** ***** ******  **** ***** * ** ************ ***  ** **** ****  *
GSTP1_Met  GGGCGGCGGCGTAGCGGTTCGTGGTTAGAGTCGTGATTTAGTACGGGGGGCGGGTCGGTTAGTCGAGTCGCGTTT
GSTP1_db   GGGCGGCGGCGCAGCGGCTCGTGGCCAGAGCCGTGACTCAGCACGGGGGGCGGGCCGGCCAGCCGAGCCGCGCCT

           *************************   * ** ******* * *** *
GSTP1_Met  GGCGGGGAGAGGCGGGGCGGGGCGCGTTTATCGTTATAAGGTTTTAGGTC
GSTP1_db   GGCGGGGAGAGGCGGGGCGGGGCGCGCCCACCGCTATAAGGCTCAGGCC
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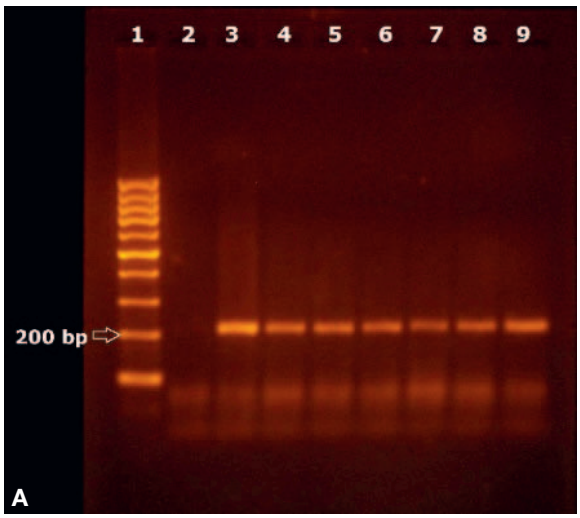
Fig. 1. Alignment of the sequences of a 200 bp band amplified with primers for the methylated bovine *GSTP1* gene and the fully methylated bisulphite-modified bovine DNA as the template (GSTP1_Met), with the original *GSTP1* sequence from the ENSEMBL database (GSTP1_db). The green spots indicate the non-CpG cytosines in the original *GSTP1* sequence that were bisulphite-converted to uracils/thymines and the yellow ones designate the CpG dinucleotides with the non-converted methylated cytosines. The stars indicate the consistent sequences between both strands.



In contrast to the MSP analysis with primers for the unmethylated *GSTP1* gene, analysis of the same samples with primers for the methylated gene did not indicate the presence of the expected 200 bp band in any of the samples, except in the sample where fully methylated standard bovine DNA was used as the template (Fig. 3A). The 200 bp band was not present in the bisulphite-modified standard bovine DNA sample presumed to be unmethylated (results not shown).

In the amplification profiles obtained with both primers for the methylated and unmethylated *GSTP1* gene, some non-specific bands below 100 bp in size were also present (Figs. 2A, 3A).

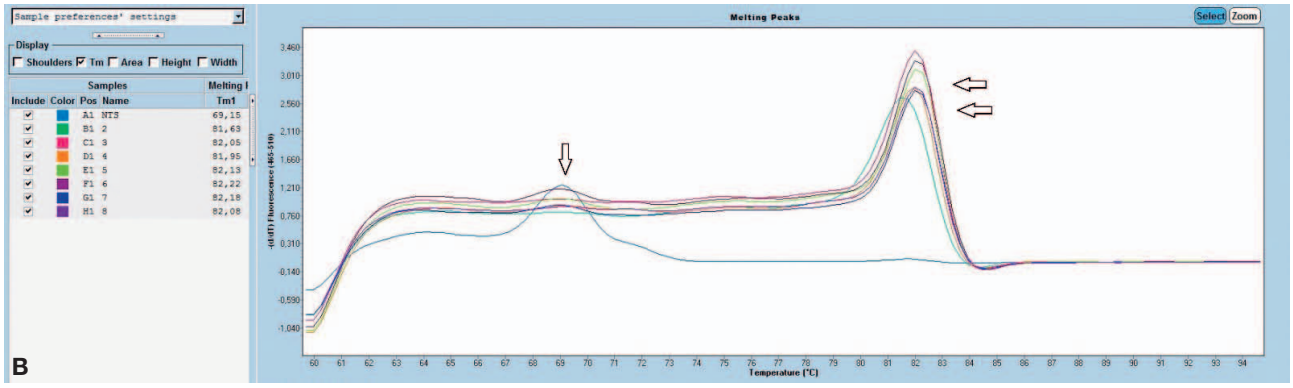


Fig. 2. **A –** Electrophoretic analysis of amplification mixtures obtained with MSP primers for the unmethylated *GSTP1* gene and the following bisulphite-modified bovine lymphocyte DNAs of one of two animals after treating with the mixtures of pesticides Mospilan 20SP and Orius 25EW: **1)** 100 bp DNA ladder (SBS); **2)** NTS; **3)** standard bovine DNA; **4)** untreated lymphocyte DNA; **5)** M 1.25 μg.ml^{-1}/O 1.5 μg.ml^{-1}; **6)** M 2.5 μg.ml^{-1}/O 3 μg.ml^{-1}; **7)** M 5 μg.ml^{-1}/O 6 μg.ml^{-1}; **8)** M 10 μg.ml^{-1}/O 15 μg.ml^{-1}; **9)** M 25 μg.ml^{-1}/O 30 μg.ml^{-1}. The blue arrow indicates a 209 bp band **B** – The output (melting curves) of the real-time MSP analysis of the same samples as in A: **A1** NTS; **B1** standard bovine DNA; **C1** untreated lymphocyte DNA; **D1** M 1.25 μg.ml^{-1}/O 1.5 μg.ml^{-1}; **E1** M 2.5 μg.ml^{-1}/O 3 μg.ml^{-1}; **F1** M 5 μg.ml^{-1}/O 6 μg.ml^{-1}; **G1** M 10 μg.ml^{-1}/O 15 μg.ml^{-1}; **H1** M 25 μg.ml^{-1}/O 30 μg.ml^{-1}. NTS = non-template sample; M = Mospilan 20SP; O = Orius 25EW. The expected peak corresponding to a 209 bp band that is present in samples B1, C1, D1, E1, F1, G1, and H1, but not in sample A1 (NTS), is indicated by a double arrow. The peak present in sample A1 represents a non-specific product and is indicated by a single arrow.

Comparison of MSP with its real-time version followed by a melting procedure

The effect of the combination of pesticides Mospilan 20SP and Orius 25EW on the methylation status of the bovine *GSTP1* gene was studied using its real-time version followed by a melting procedure in addition to MSP analysis.

In initial real-time MSP amplifications with primers for the unmethylated and methylated *GSTP1* gene, we used the same annealing temperatures as in MSP amplifications (both 54 °C and 55 °C), and we tried higher annealing temperatures – 60 °C in reactions with primers for the unmethylated *GSTP1* gene and 61 °C in reactions with primers for the methylated *GSTP1* gene. However, we did not find any significant differences when comparing amplification and melting profiles generated at higher annealing temperatures with those at lower temperatures (results not shown). So, for further real-time MSP and melting analysis, we used the same annealing temperatures as those for MSP for both sets of primers.

In the melting profiles of PCR products obtained by real-time amplification with primers for the unmethylated as well as methylated *GSTP1* gene, it was possible to identify peaks corresponding to the specific bands in the electrophoretic profiles obtained by MSP analysis. The 209 bp band, which was the result of MSP with primers for the unmethylated *GSTP1* gene, corresponded to a distinct peak with a height at approximately 82.5 °C (Fig. 2B). The 200 bp band to be amplified by MSP with primers for the methylated *GSTP1* gene corresponded to the peak with a height at approximately 84 °C (Fig 3B). Real-time MSP analysis followed by a melting procedure provided concordant results with the results of MSP analysis – the specific peak corresponding to the expected 209 bp band (indicated by a double arrow) was present in the melting profiles of the amplification products obtained with primers for the unmethylated *GSTP1* gene of standard bovine DNA and DNA from lymphocytes of both animals untreated and treated with all amounts of Mospilan 20SP alone and in combination with Orius 25EW (Fig. 2B). Real-time MSP followed by a melting procedure with primers for the methylated *GSTP1* gene of the same samples as with primers for the unmethylated gene showed the absence of the expected peak corresponding to the specific 200 bp band (Fig. 3B). As expected, a peak corresponding to the specific 200 bp band (indicated by a double arrow) was present only in the sample where fully methylated standard bovine DNA was used as a template.

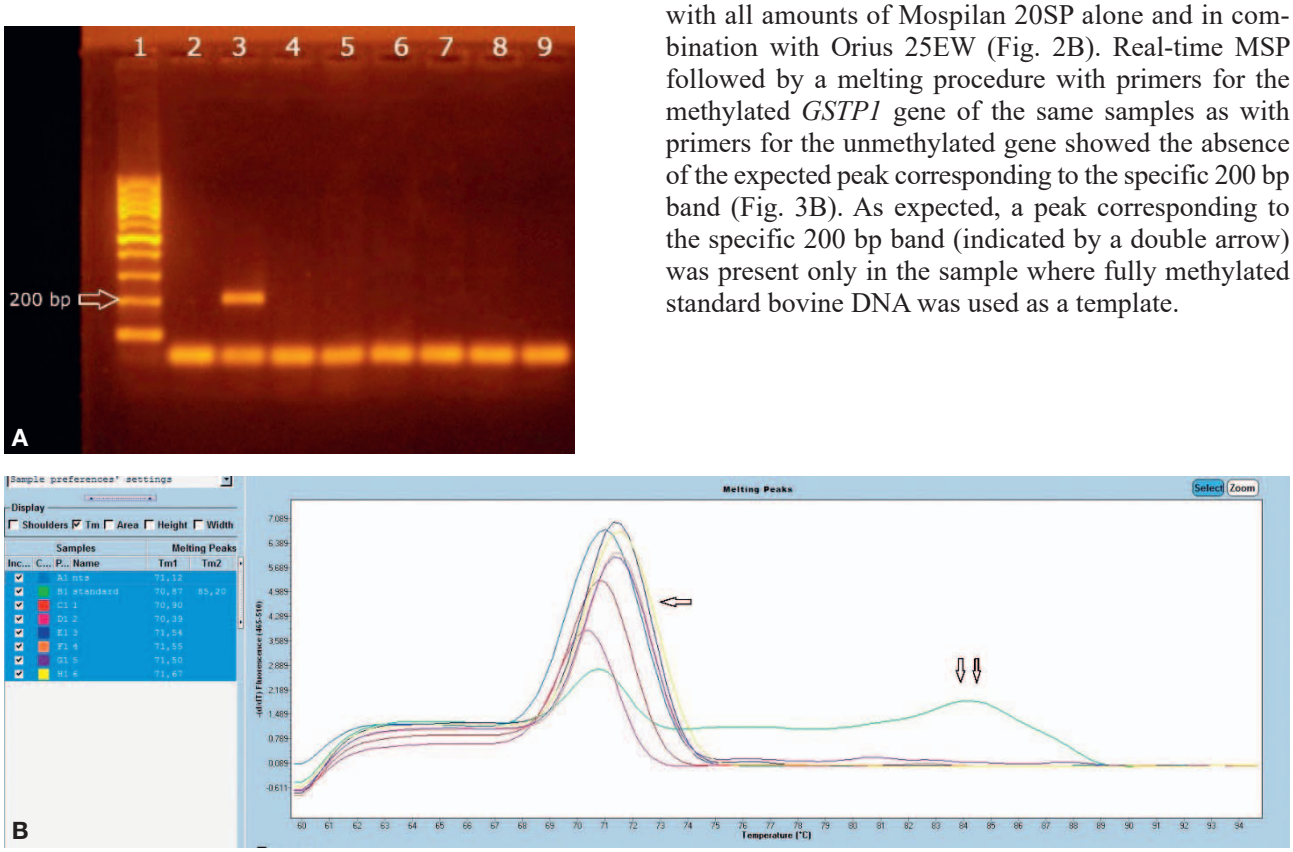


Fig. 3. **A** – Electrophoretic analysis of amplification mixtures obtained with MSP primers for the methylated *GSTP1* gene and the following bisulphite-modified bovine lymphocyte DNAs of one of two animals after treating with the mixture of pesticides Mospilan 20SP and Orius 25EW: **1)** 100 bp DNA ladder (SBS); **2)** NTS; **3)** fully methylated standard bovine DNA; **4)** untreated lymphocyte DNA; **5)** M 1.25 μg.ml^{-1}/O 1.5 μg.ml^{-1}; **6)** M 2.5 μg.ml^{-1}/O 3 μg.ml^{-1}; **7)** M 5 μg.ml^{-1}/O 6 μg.ml^{-1}; **8)** M 10 μg.ml^{-1}/O 15 μg.ml^{-1}; **9)** M 25 μg.ml^{-1}/O 30 μg.ml^{-1}. **B** – The output (melting curves) of the real-time MSP analysis of the same samples as in A: **A1** NTS; **B1** fully methylated standard bovine DNA; **C1** untreated lymphocyte DNA; **D1** M 1.25 μg.ml^{-1}/O 1.5 μg.ml^{-1}; **E1** M 2.5 μg.ml^{-1}/O 3 μg.ml^{-1}; **F1** M 5 μg.ml^{-1}/O 6 μg.ml^{-1}; **G1** M 10 μg.ml^{-1}/O 15 μg.ml^{-1}; **H1** M 25 μg.ml^{-1}/O 30 μg.ml^{-1}. NTS = non-template sample; M = Mospilan 20SP; O = Orius 25EW. The expected peak corresponding to a 200 bp band that is present in the B1 sample only is indicated by a double arrow. A single arrow indicates a non-specific peak that is present in all samples, including NTS.

Similar to the amplification profiles obtained by MSP, we identified some non-specific products in the outputs of the melting analysis. These were peaks with heights at approximately 69.5 °C (for primers for the unmethylated *GSTP1* gene, Fig. 2B) and 71 °C (for primers for the methylated *GSTP1* gene, Fig. 3B).

Discussion

The present work aimed to investigate whether the pesticide Mospilan 20SP alone and in combination with the pesticide Orius 25EW can induce methylation of the bovine *GSTP1* gene in *in vitro* proliferating bovine lymphocytes. As far as we know, only one study has been published to date that analysed the effect of a pesticide on the methylation status of a bovine gene. Pallotta et al. (2019) revealed methylation changes in one region of imprinted *XIST* promoter in bovine spermatozoa treated with 10 $\mu g.ml^{-1}$ of organophosphate pesticide chlorpyriphos. In another study, not DNA methylation but histone methylation in bovines was investigated. Pang et al. (2018) found that melatonin caused a reversal in the decline of bovine oocyte maturation induced by broad-spectrum agricultural pesticide paraquat via inhibition of an increase of the trimethyl-histone H3 lysine 4 (H3K4me3) and a decrease of the trimethyl-histone H3 lysine 9 (H3K9me3) level.

In our study, by validating primers previously designed for MSP analysis of the methylation status of the *GSTP1* gene, we confirmed that they are correct. Although MSP is a relatively simple and useful method for assessing the methylation status of promoter or exon regions of genes, a certain drawback is a need to detect amplification products in agarose gel. Therefore, we also focused on the use of real-time MSP with SYBR Green intercalation dye followed by a melting procedure. The results of this analysis, represented by the melting profiles of the real-time amplification products, corresponded with the electrophoretic profiles obtained by MSP analysis. Moreover, the progression of the curves generated with standard unmethylated and methylated DNA as templates allowed the methylation status of the samples analysed to be detected by comparison with them.

We assumed that if Mospilan 20SP alone or in combination with Orius 25EW caused methylation of the *GSTP1* gene, we would identify both the expected 200 bp band in the electrophoretic profile and the corresponding specific peak in the melting output generated with primers for the methylated *GSTP1* gene in some of the DNA samples isolated from lymphocytes cultured with pesticides. However, we did not identify this expected band or corresponding peak in any such sample at all for the quantities of Mospilan 20SP alone and in combination with Orius 25EW and all times of exposure used. On the contrary, in the amplification profiles and melting outputs of pesticide-treated and untreated samples as well as of standard DNA, we found an expected 209 bp band and an appropriate specific peak that should be generated with primers for the unmethylated *GSTP1* gene. So, our results indicate that the *GSTP1* gene was probably unmethylated both in the pesticide-unexposed and pesticide-exposed samples.

Both in the amplification profiles obtained by MSP and in the outputs of melting of real-time amplification products, we identified some non-specific bands and peaks in addition to the specific ones with both sets of primers. These undesired bands and peaks, which probably arise as a result of auto-amplification of primers, were also identified in the NTS sample, confirming their non-specificity. According to Šestáková et al. (2019), finding convenient methylated and unmethylated primers is sometimes challenging. We must emphasize that designing these primers in a given CpG-rich region of the specific gene requires taking into account many requirements. Therefore, it is unavoidable that primers meeting the given requirements show amplification of certain non-specific products even in the absence of template DNA. In such a case, the inclusion of NTS and standard unmethylated and methylated DNA, against which the samples analysed can be compared, should be a sufficient measure to obtain reliable results.

We can conclude that the pesticide Mospilan 20SP alone as well as in combination with the pesticide Orius 25EW did not cause changes in terms of induction of methylation in the bovine *GSTP1* gene in *in vitro* proliferating bovine lymphocytes. We also found out that the less laborious real-time MSP followed by a melting procedure, which does not require the detection of amplification products in agarose gel, may replace MSP for studying the methylation status of the bovine *GSTP1* gene.

However, since the MSP allows analysis of the methylation of only those CpG dinucleotides that are part of primer sequences, in the future, additional CpG dinucleotides in other regions of promoter or first exon of the bovine *GSTP1* gene will need to be analysed, either by designing other primers or by the use of bisulphite sequencing. Moreover, the influence of these and other pesticides on methylation of some other members of the *GST* gene family will be investigated. Last but not least, the study of a possible pesticide impact on the methylation of various genes underlying the economically important cattle traits such as meat or milk quality and production would be of great importance.

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