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Short Communication

Optimizing single-tube nested PCR for enhanced genotyping of single cells and *in vitro* produced bovine embryos

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ABSTRACT

Single-tube nested PCR (STnPCR) is a technique that improves nested PCR, reducing potential contamination and false-positive results, enhancing the amplification sensitivity. Despite being commonly used for the detection of microorganisms, STnPCR can be a valuable tool for bovine genotyping, encompassing essential targets as *ROSA26* and *TSPY*, pivotal in the fields of animal reproduction, genetic improvement, and transgenic research. The objective of this study was to improve and innovate STnPCR for gene detection in cattle. We aimed to detect the *ROSA26* and *TSPY* genes using low-concentration DNA samples, including single cells, small cell groups (one to five cells), *in vitro*-produced embryos, and bovine tissue samples. Moreover, we refined STnPCR for gene detection in up to single cells by conducting sensitivity testing with different concentration ratios of internal and external primers. Successful amplification of the *ROSA26* and *TSPY* genes was achieved across all tested primer concentrations, even in single cells, with more consistent results observed at lower primer concentrations. Additionally, simultaneous gene amplification was achieved through STnPCR multiplexing, representing the first study of multiplex STnPCR in cattle. These outcomes not only confirm its effectiveness in detecting genetic markers for animal genetic improvement and transgenic elements but also pave the way for its widespread adoption in reproductive studies in bovines.

1. Introduction

Since its inception, PCR has undergone significant advancements, finding numerous applications in science. Over the years, it has enabled various applications, such as the analysis of genetic material from individual cells and has emerged as the preferred method for evaluating nucleic acids in molecular diagnostics, providing reliable qualitative and quantitative results (Zhu et al., 2020). One of the advantages of PCR is the amplification of genetic material at low concentrations, as seen in nested PCR (nPCR) which increases the sensitivity and specificity offering a cost-effective alternative to other techniques (Green and Sambrook, 2019).

Developed to improve the sensitivity of target sequence detection, nPCR relies on two amplification steps. The first amplification generates a larger sequence, and the second PCR amplifies an internal sequence within the product of the first amplification (Shatleh-Rantisi et al., 2020). Internal primers are used to boost specificity, reducing the chance of nonspecific amplification, while employing two pairs of

Abbreviations: °C, degree celsius; µg, microgram; µl, microliter; µM, micromolar; bp, base pair; Cas, CRISPR associated proteins; cDNA, complementary DNA; CRISPR, Clustered Regularly Interspaced Short Palindromic Repeats; ddH2O, Double-distilled water; DNA, Deoxyribonucleic acid; dNTP, deoxyribonucleoside triphosphate; ELISA, Enzyme-Linked Immunosorbent Assay; FBS, Fetal Bovine Serum; fg, femtogram; FSH, Follicle Stimulating Hormone; LH, Luteinizing Hormone; ml, milliliter; mm, millimeter; mM, millimolar; ng, nanogram; nPCR, nested PCR; PCR, Polymerase Chain Reaction; pg, picogram; RFQ-PCR, Real-time Fluorescence based Quantitative – PCR; RNA, Ribonucleic acid; RT-LAMP, Reverse Transcription – Loop Mediated Isothermal Amplification; RT-qPCR, Reverse Transcription-quantitative PCR; SDS, Sodium Dodecyl Sulfate; STnPCR, single tube nested PCR; TSPY, Testis-specific protein Y-encoded.

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primers heightens sensitivity by amplifying the target DNA region twice (Deepachandi et al., 2019). However, despite its sensitivity benefits, careful adjustment of parameters such as primer concentrations and reaction setup are crucial to prevent contamination, which can lead to false-positive results (Deepachandi et al., 2019). Exposure of initial reaction materials to the external environment risks compromising DNA integrity, potentially impacting subsequent reactions (Abath et al., 2002). Despite its advantages, the nPCR technique remains vulnerable to contamination during the amplification steps.

To address contamination concerns and false-positive amplifications, a new methodology was developed: single-tube nested PCR (STnPCR) (Deepachandi et al., 2019), which performs amplification with both outer and inner primers within a single reaction tube (Hamim et al., 2018). Commonly used in microorganisms' detection (da Costa-Lima et al., 2020; Shatleh-Rantisi et al., 2020; Wang et al., 2020), STnPCR builds upon nested PCR with more sensitivity (Abath et al., 2002). To optimize STnPCR for low-concentration samples like single cells, it's crucial to ensure that the initial round of amplification fully utilizes the concentration of primers targeting the outer regions, depleting them by the end of the first PCR. These features make STnPCR a valuable option for research and diagnostics requiring accurate and sensitive detection of specific DNA sequences (Deepachandi et al., 2019).

STnPCR technology, despite its broad applicability (Lima et al., 2015; Pontes et al., 2014; Souza et al., 2007; Zhang et al., 2023), is underutilized in domestic animal genotyping, notably in bovine species. Its adoption could enhance bovine genotyping, offering faster analysis and reduced costs, holding a promise for identifying molecular markers and detecting transgenes post-gene editing. Additionally, STnPCR can analyze various single cells, enabling the identification of genetic markers associated with traits such as milk production, disease resistance, and morphology.

This study utilized STnPCR to optimize bovine gene detection, focusing on two genes: *ROSA26* and *TSPY*. *ROSA26* serves as a stable genetic marker essential for animal reproduction and transgenics (Wang et al., 2018; Xie et al., 2022; Yuan et al., 2021; Zhang et al., 2021). *TSPY*, located on the Y chromosome, is used for early embryo sex identification, and plays a role in male gamete development (Oluwole et al., 2017; Rho et al., 2023; Schnieders et al., 1996). In addition to using samples with low DNA concentrations, the study aimed to optimize the technique by exploring various combinations of primer concentrations. The findings from this research endeavor are expected to contribute to a more accurate and reliable genetic characterization of bovine embryos, advancing knowledge in animal reproduction.

2. Materials and methods

2.1. Samples

Initially, the study evaluated whether the STnPCR technique would be able to detect/amplify genes in a single diploid cell (sensitivity test). To achieve this, the samples consisted of single cells, as well as sets of cells grouped into 2, 3, 4, and 5-cell clusters. Additionally, bovine liver tissue samples at concentrations of 0.2 ng, 2 ng and 10 ng, were included along with bovine embryos (blastocysts − D7) produced *in vitro*.

The groups of cells derived from ovaries and the bovine liver samples were obtained from a slaughterhouse, immediately after the animals were abattoir. The collection of biological material followed the routine procedures of the official slaughterhouse. Both types of samples were then transported to the laboratory in a 0.9 % saline solution and 1 % penicillin/streptomycin. For the set of grouped cells, naked oocytes (without cumulus cells) were used in groups of samples consisting of 1, 2, 3, 4 and 5 cells. Cumulus-oophorus complexes (COCs) were aspirated from the ovaries using a syringe and a 19G needle (40 x 1.2 mm). After selection, the COCs were washed in 100 μl drops of medium-199-HEPES supplemented with 10 % fetal bovine serum and 1 % penicillin/streptomycin. Selected COCs (n = 1, 2, 3, 4, 5) were stripped by pipetting and collected in a microtube containing 2 μl of medium-TCM199-hepes and 5 μl of RNAlater and stored at −20 °C until DNA extraction.

2.2. In vitro bovine embryo production

Ovaries were obtained from cows from local slaughterhouses and transported to the laboratory in containers at 38 °C, with 0.9 % saline solution supplemented and 10 μl/ml of penicillin/streptomycin. Aspiration of the follicles was performed using a 10 ml syringe and a 19 G needle (40 x 1.2 mm). Only aspirated oocytes with more than two layers of cumulus cells and uniform cytoplasm were selected. The selection process occurred in a petri dish with TCM199-hepes medium at 35 °C. Approximately 30 selected oocytes were transferred to 100 μl drops of maturation medium, in 35 mm plates containing medium-TCM199, 10 % fetal bovine serum (FBS), 0.5 μg/ml follicle stimulating hormone (FSH), 5 μg/ml luteinizing hormone (LH), 10 μl/ml of penicillin/streptomycin, 0.2 mM pyruvate, and 0.4 mM glutamine. The oocytes were incubated for 22 h at 38.5 °C and 6 % CO_2.

After *in vitro* maturation, the matured oocytes were fertilized with cryopreserved semen. The semen was thawed at 37 °C for 30 s and processed using a miniPercoll gradient (Percoll 90 % / 45 %). The inseminating dose of 2 x 10^6 sperm was co-incubated with oocytes in Talp-fec fertilization medium supplemented with PHE (0.8 % saline solution, 2 mM penicillin, 1 mM hypotaurine, 250 μM epinephrine) and 10 μg/ml heparin. This co-incubation was carried out at 38.5 °C and 6 % CO_2 for 18 h.

For *in vitro* culture, presumptive zygotes were denuded and incubated in SOF medium supplemented with 2.5 % FBS, 0.5 mM Tri-sodium citrate, 2.77 mM Myo-inositol, 20 μl/ml BME essential, 10 μl/ml MEM non −essential, 1 μl/ml penicillin/streptomycin, 100 mM sodium pyruvate and 100 mM glutamine. The zygotes were then incubated for 7 days at 38.5 °C and 5 % CO_2. On day 7, the resulting blastocysts were collected, stored in 10 μl of RNAlater at −20 °C, and kept until DNA extraction.

2.3. DNA extraction

Immediately after collecting the material, 1 μl proteinase K (25 mg/ml) and 49 μl 0.005 % SDS were added (Carneiro et al., 2011). The microtubes were incubated at 55 °C for 1 h and at 99 °C for 30 min. The samples were stored in the −20 °C freezer until STnPCR analysis. The single cell sample, the groups of cells (from 2 to 5), and the embryos produced *in vitro* (D7) were stored at −20 °C until the moment of analysis, the same as recommended by (Carneiro et al., 2011). Only the bovine liver tissue samples were quantified by reading on a NanoDrop OneC spectrophotometer, with samples presenting values of 1.8 to 2.0 at an optical density of 260/280 being considered of good quality.

2.4. Primer design

All primers used for STnPCR protocol were designed using sequences from the bovine reference genome bosTau7, obtained from the UCSC Genome Browser. The software Primer-BLAST (https://www.ncbi.nlm.nih.gov/tools/primer-blast/) was used to design the primers. The chosen regions of *ROSA26* and *TSPY* bovine genes are described in Supplementary table 1.

2.5. STnPCR protocol (single tube nested PCR)

The STnPCR protocol was performed according to the previous description in the literature (Haff, 1994; Yourno, 1992; Pontes et al., 2014; Lima et al., 2015) (Fig. 1). For the first step of STnPCR, different concentrations of the inner primers (0.2 μM; 0.5 μM) were added into the open lid of the 0.2 ml microtube with 0.5 μl bromophenol 6x for 30 min at 37 °C in the thermal cycler for drying and adherence of the internal primers to the lid. The thermocycler lid temperature was 10 °C higher

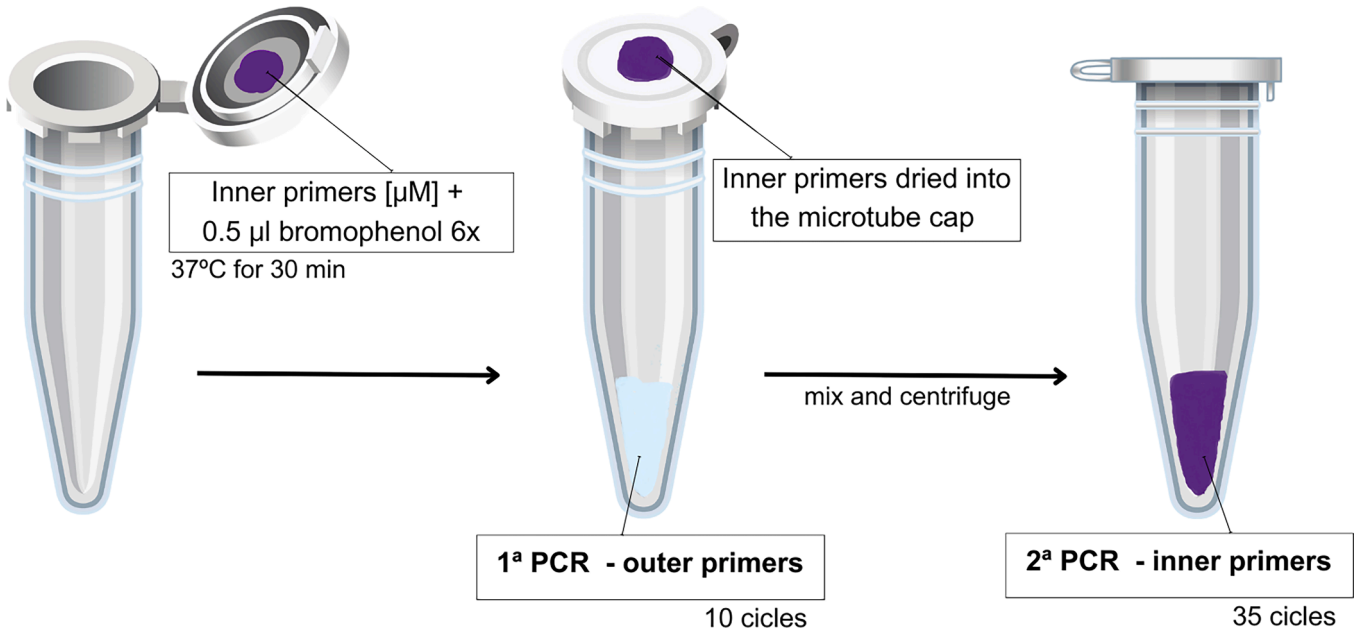

Fig. 1. STnPCR methodology. Step-by-step schematic representation of the amplification process of *ROSA26* and *TSPY* genes in samples of 1, 2, 3, 4, and 5 cells, liver tissue samples with 10 ng, 2 ng, and 0.2 ng, and *in vitro* embryo produced (blastocysts – D7).

than the maximum temperature used (37 °C), to prevent excessive sample evaporation. Subsequently, 1st PCR was performed with the outer primers in a final volume of 20 μl [6.76 μl ddH2O; 4.0 μl 5x Buffer; 0.2 μM / 0.1 μM / 0.05 μM / or 0.01 μM of each outer primer (sense and antisense); 0.25 mM of each dNTP; 0.2 μl Phusion High Fidelity DNA Polymerase Thermo Scientific; 8.0 μl of DNA). The cycling conditions

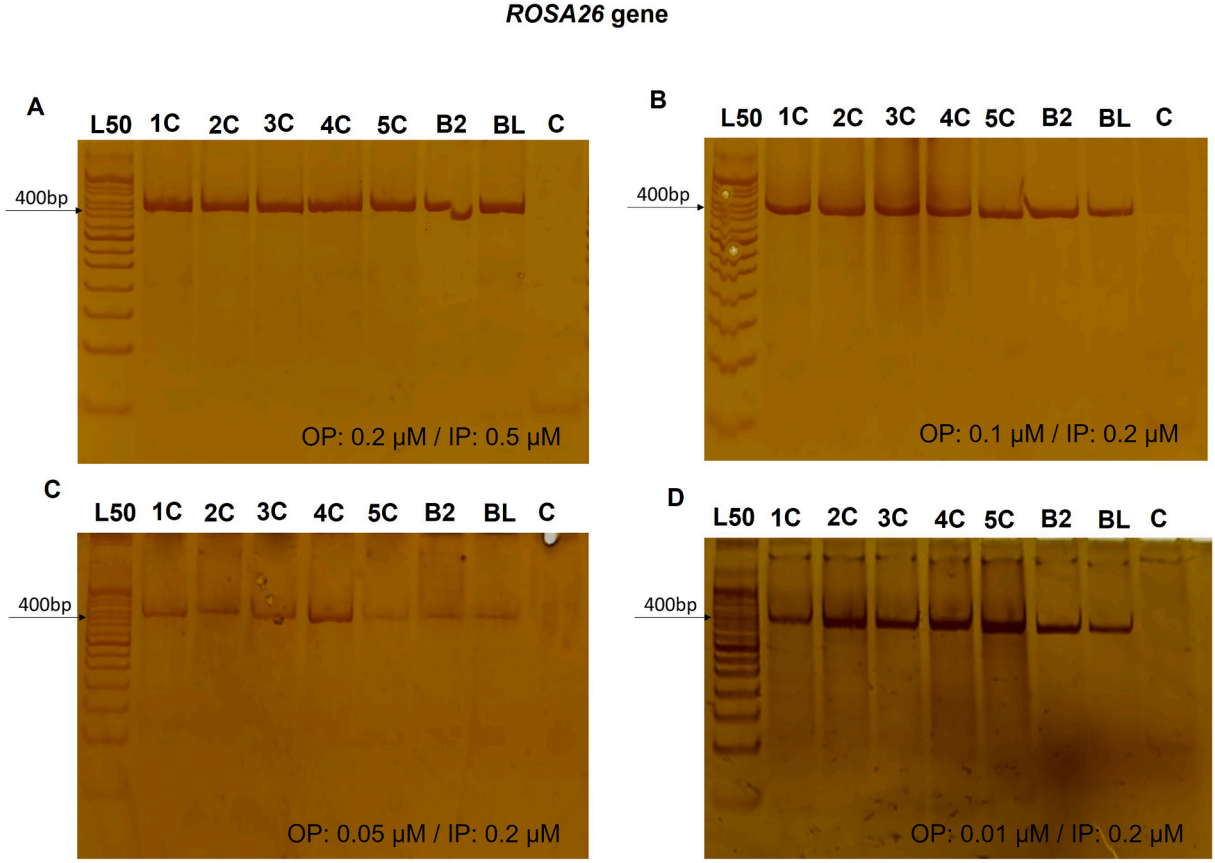

Fig. 2. STnPCR protocol of bovine *ROSA26* gene. Evaluation of *ROSA26* region (435 bp) amplification in different primers concentrations. (A) OP: 0.2 μM / IP: 0.5 μM; (B) OP: 0.1 μM / IP: 0.2 μM; (C) OP: 0.05 μM / IP: 0.2 μM; (D) OP: 0.01 μM / IP: 0.2 μM. Samples: single cell (1C), two cells (2C), three cells (3C), four cells (4C), five cells (5C), bovine tissue in 2 ng concentration (B2), and *in vitro*-produced blastocysts (BL), PCR negative control (C). 12 % acrylamide gel stained with 0.2 % silver nitrate. *L50: Ladder 50pb / OP: outer primer / IP: inner primer.

were 98 °C for 2 min, 10 cycles of 98 °C for 30 s, annealing temperature (°C) for 30 s (Supplementary table 2) and 72 °C for 30 s. After the 1st PCR, the microtubes were incubated at 98 °C for approximately 1 min in the thermocycler. Following this, they were inverted several times to release the inner primers from the lid into the reaction mixture, briefly centrifuged for 1 min at 6000 x g, and then returned to the thermocycler for the second round of amplification. For the 2nd PCR and amplification of the inner primer region, the cycling conditions were: 98 °C for 2 min, 35 cycles of 98 °C for 30 s, annealing temperature (°C) for 30 s (Supplementary table 2), and 72 °C for 30 s, with a final extension of 72 °C for 10 min. PCR products were visualized on a 12 % acrylamide gel stained with 0.2 % silver nitrate. The concentration combinations of outer and inner primers used in the 1st and 2nd PCR, respectively, are described in Supplementary table 2.

3. Results

3.1. ROSA26 and TSPY gene detection and primer sensitivity evaluations

ROSA26 gene amplification was observed in samples with low DNA concentration, such as single cells and *in vitro*-produced embryos (D7 blastocysts), by evaluating different concentrations of outer and inner primers for the gene. Amplification of *ROSA26* was observed for all combinations of outer and inner primer concentrations (Fig. 2). In Fig. 2A, when using higher concentrations of outer (0.2 μM) and inner (0.5 μM) primers, all samples exhibited amplification of the *ROSA26* gene. Similarly, the same amplification pattern was observed when decreasing the primer concentrations. Even at the lowest concentration of outer primer (0.05 μM and 0.01 μM), amplification in the expected position was observed for samples from cell groups (1C to 5C), bovine tissue in 2 ng concentration of DNA, and blastocysts (Fig. 2B-D).

The *TSPY* gene results revealed successful amplifications across various concentrations of both outer and inner primers (Fig. 3). The amplified 157 bp fragment was present in all samples and primer concentrations tested. Male bovine tissue was chosen as the sample since the *TSPY* gene is a specific marker of chromosome Y. Similarly to the *ROSA26* gene, even with a decrease in the concentration of outer primers (0.05 μM and 0.01 μM) (Fig. 3C-D), it was possible to observe that all samples amplified without nonspecific amplifications, indicating that the parameters were consistent and robust.

3.2. Multiplex STnPCR analysis of ROSA26 and TSPY gene

For the simultaneous amplification of the *ROSA26* and *TSPY* genes, a

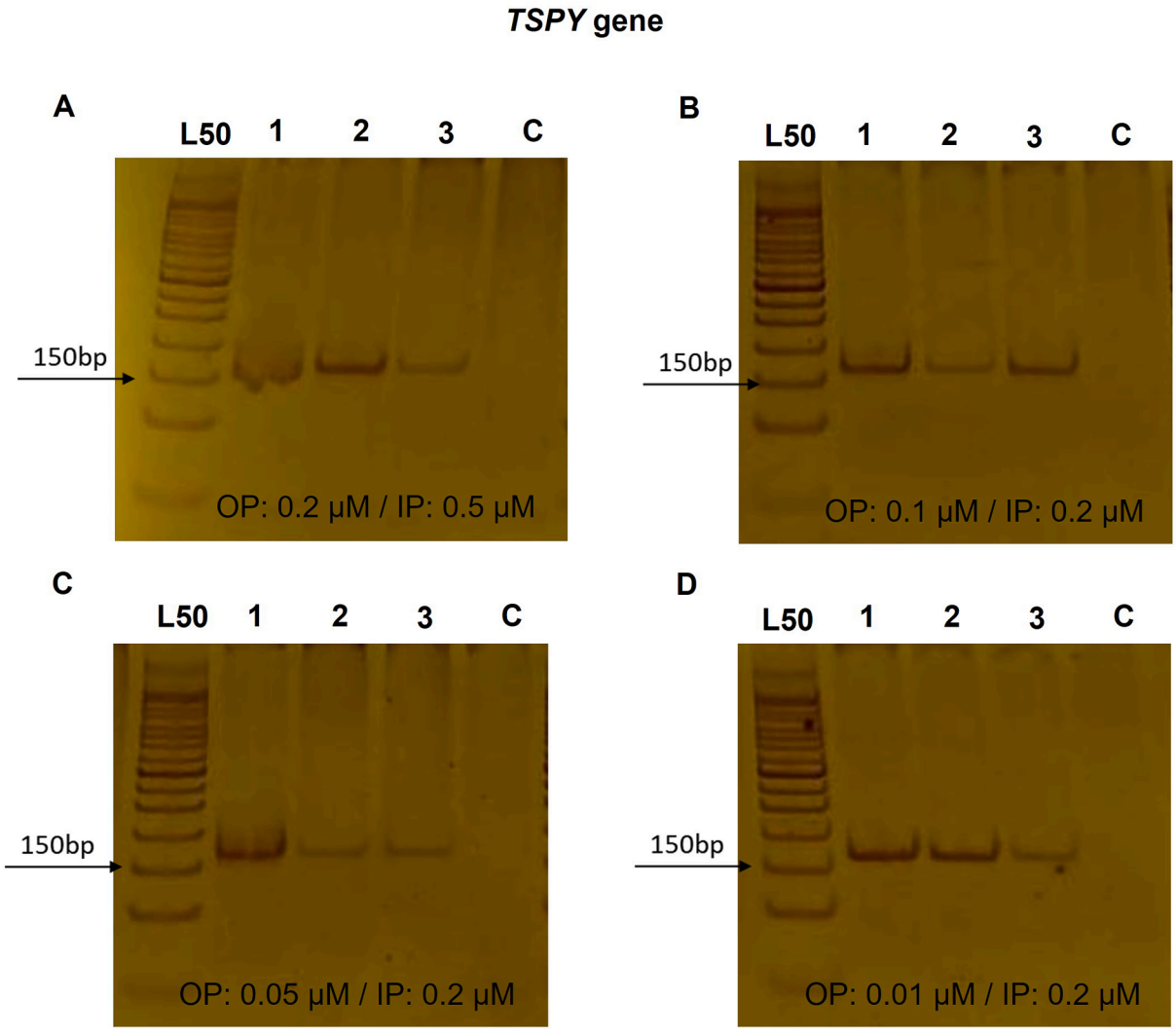

Fig. 3. STnPCR protocol of bovine *TSPY* gene. Evaluation of *TSPY* region (157 bp) amplification in different primers concentrations. (A) OP: 0.2 μM / IP: 0.5 μM; (B) OP: 0.1 μM / IP: 0.2 μM; (C) OP: 0.05 μM / IP: 0.2 μM; (D) OP: 0.01 μM / IP: 0.2 μM. Samples: 1. bovine tissue in 10 ng concentration, 2. bovine tissue in 2 ng concentration, 3. bovine tissue in 0.2 ng concentration, C. PCR negative control. 12 % acrylamide gel stained with 0.2 % silver nitrate. *L50: Ladder 50pb / OP: outer primer / IP: inner primer.

different combination of external primer concentrations (*ROSA26* – 0.05 μM and *TSPY* – 0.01 μM) were utilized (Fig. 4). The *ROSA26* gene employed an outer and inner primer concentration of 0.05 μM and 0.2 μM, respectively. On the other hand, the *TSPY* gene utilized a concentration of 0.01 μM for outer primers and 0.2 μM for inner primers. These concentrations were selected based on the proximity of the primer annealing temperature (Supplementary table 2). Notably, all bovine tissue samples, even in the lowest concentration (0.2 ng), exhibited successful amplification for both regions (*ROSA26* – 435 bp and *TSPY* – 157 bp).

4. Discussion

STnPCR stands as a significant advancement over conventional nPCR, mainly due to its ability to substantially reduce cross-contamination between the two PCR reaction rounds. Alongside this critical benefit, STnPCR offers numerous other advantages, including efficient sample utilization, reduced reagent consumption, and faster analysis times. Nonetheless, this technique presents certain limitations, particularly concerning the design of outer and inner primers and the necessity for primers with identical annealing temperatures for multiplex reactions.

Initially, the study aimed to detect the *ROSA26* and *TSPY* regions in samples with limited amounts of DNA, such as single cells and *in vitro* produced bovine embryos. The ratios of inner to outer primers used in this study were based on those reported in the literature, where some studies utilized a 10:1 (inner:outer) ratio (Abath et al., 2002; Pontes et al., 2014) and others up to 50:1 (Deepachandi et al., 2019). In our study, we selected a broader concentration range to test different ratios, including 1:1 (0.2 μM inner primer: 0.2 μM outer primer), 10:1 (0.5 μM inner: 0.05 μM outer), and even 50:1 (0.5 μM inner: 0.01 μM outer) (Supplementary table 1).

Successful *ROSA26* gene amplification was achieved across all tested primer concentration combinations, including extremely low levels such as 0.05 μM and 0.01 μM, in various sample types, such as single cells, bovine tissue, and blastocysts (Fig. 2). Studies utilizing the *ROSA26* gene as a transgene integration site have successfully genotyped the region using various techniques, including RT-qPCR, conventional PCR, RT-PCR, qPCR, and nested PCR (Liu et al., 2018; Wang et al., 2018; Yang et al., 2016). Our findings suggest that STnPCR shows promise for transgene detection and genotyping in gene editing protocols.

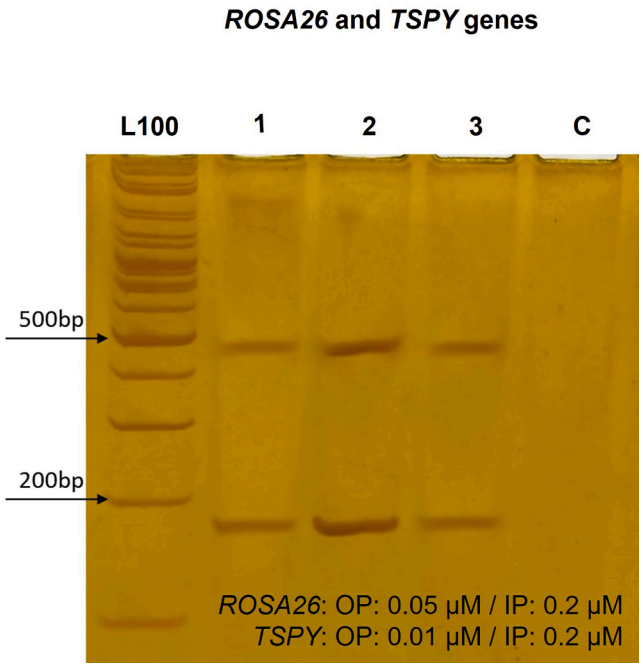

Fig. 4. Multiplex STnPCR of bovine *ROSA26* and *TSPY* genes. Simultaneous amplification of *ROSA26* (435 bp) and *TSPY* (157 bp) in different primer concentration. *ROSA26* gene: OP: 0.05 μM / IP: 0.2 μM. *TSPY* gene: OP: 0.01 μM / IP: 0.2 μM; Samples: 1. bovine tissue in 10 ng concentration, 2. bovine tissue in 2 ng concentration, 3. bovine tissue in 0.2 ng concentration, C. PCR negative control. 12 % acrylamide gel stained with 0.2 % silver nitrate. * L50: Ladder 50pb / OP: outer primer / IP: inner primer.

TSPY gene amplification was successful across various primer concentrations without non-specific amplifications, demonstrating parameter reliability (Fig. 3). Genotyping of the *TSPY* gene can be performed using techniques such as quantitative PCR and real-time fluorescence-based quantitative PCR (RFQ-PCR) (Hamilton et al., 2009; Su et al., 2006; Vodicka et al., 2007; Yasmin et al., 2016). STnPCR for *TSPY* detection offers time and resource savings by facilitating rapid identification and disposal of unwanted sexual embryos, contributing to gender selection programs.

To simultaneously amplify both regions, even at very low concentrations of genetic material (0.2 ng), we successfully amplified *ROSA26* and *TSPY* in all bovine tissue samples at the correct positions (Fig. 4). For the multiplex STnPCR of *ROSA26* and *TSPY*, we strategically chose to use different concentrations of outer primers due to their similar annealing temperatures. However, for the inner primers, despite using the same concentration (0.2 μM), we adjusted the *TSPY* annealing temperature from 64 °C to 62 °C to match that of the *ROSA26* gene (Supplementary table 2). After making the necessary adjustment, we were pleased to observe successful amplification of both regions.

One of the limitations in performing a multiplex STnPCR is designing primers with a suitable annealing temperature for all the primer pairs used (Mendes et al., 2008). This factor is crucial because the annealing temperature directly impacts amplification specificity. If the temperature is too low, the primers may bind to non-specific sequences, while excessively high temperatures can result in insufficient binding between the primer and the complementary strand, leading to decreased amplification efficiency (Lorenz, 2012).

Another limitation factor in STnPCR is the combination of outer and inner primer concentrations, which should be chosen based on a difference of approximately 5 °C in their annealing temperatures (Lorenz, 2012). According to a prior study, it is preferable for the annealing temperature of the internal primers to be 10 °C higher than that of the external primers, as it enhances the amplification efficiency (Hamim et al., 2018). In the present study, although the difference in concentrations between outer and inner primers ranged from 3 °C to 7 °C, specific amplification for both analyzed regions was successfully achieved.

In summary, STnPCR demonstrated 100 % sensitivity and specificity in diagnosing microorganisms, without cross-contamination (Deepachandi et al., 2019). Compared to Real-time PCR, STnPCR is more cost-effective and sensitive, though the choice depends on study goals and resources (Hamim et al., 2018). In the study to detect PRSV (papaya ringspot virus) in symptomatic and asymptomatic samples, STnPCR outperformed ELISA and RT-PCR, detecting cDNA dilutions up to 10^7-fold, whereas other studies observed a limit of 10^3-fold. Moreover, it detected samples as low as 100 fg per reaction, showing superiority over other methods (Hamim et al., 2018).

5. Conclusions

Although STnPCR is commonly used for the detection of microorganisms, we expect to broaden its applications through our research. Our results clear the way for the widespread adoption of this technique in various fields, such as gene editing, reproductive studies, and animal genetic improvement. We firmly believe that our findings will help unlock its potential and open new ways for research and advancements in DNA genotyping in bovines.

CRediT authorship contribution statement

Jéssica Macedo Rafael de Arruda: Writing – review & editing, Writing – original draft, Methodology, Investigation, Formal analysis. **Mariana da Silva Mendonça:** Writing – review & editing, Writing – original draft, Methodology. **Paulo Vitor Maravilha Braga:** . **Leticia dos Santos Nascimento:** Writing – original draft, Methodology, Investigation, Formal analysis. **Angelo José Burla Dias:** Writing – review & editing, Supervision, Resources, Project administration, Funding acquisition. **Álvaro Fabrício Lopes Rios:** Writing – review & editing, Supervision, Resources, Project administration, Funding acquisition. **Paula Magnelli Mangiavacchi:** Writing – review & editing, Writing – original draft, Validation, Methodology, Investigation, Formal analysis, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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This research was supported by FAPERJ (E-26/010.000.472/2017). During the preparation of this work the author(s) used chat GPT to correct the English grammar. After using this tool/service, the author(s) reviewed and edited the content as needed and take(s) full responsibility for the content of the publication.

Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.gene.2024.148838.

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