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Solving the amplification complexity of prolificacy genes in local Ewes and Does

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ABSTRACT

The rationale of this study is to overcome the complexity in optimization of the PCR protocols in an attempt to amplify specific sites on four different genes within the Growth Differentiation Factor 9 (GDF9) in ewe and doe, a required factor expressed in oocytes for folliculogenesis. This optimization is a prerequisite for improving the selection criterion for higher prolificacy in selected newly born female kids and lambs. Results showed that optimization of PCR protocols of each of the four genes within the GDF9 required different standardizations based on many factors namely, the concentrations of extracted DNA, primer volume in the PCR mix, cycling conditions, freshness of extracted DNA, re-amplification of PCR product, and concentration of the Agarose Gel for banding amplicons. In conclusion, the complex optimization showed that each of the four genes within the GDF9 required different PCR protocols.

INTRODUCTION

Growth factors synthesized by ovarian somatic cells have a direct effect on oocyte growth and function (Wigglesworth et al. 2013). The growth differentiation factor-9 (GDF9) is a protein encoded by the GDF9 gene, expressed in oocytes, and is associated with ovarian folliculogenesis (Kovanci et al. 2007; Kona et al. 2016). The GDF9 is a member of the transforming growth factor-beta (TGFβ) superfamily. It plays a key role in the development of primary ovarian follicles, and in differentiation and maturation of the oocytes (Dragovic et al. 2005; Ahlawat et al. 2015).

The GDF9 has been related to fertility due to its role in ovulation rate and in premature cessation of ovarian function (Barzegari et al. 2010; Feng et al. 2011; Souza et al. 2014). Different mutations in the GDF9 gene may cause a change in ovulation rate of ewes (Kasiriyan et al. 2011). A study by Feng et al. in 2011 provided the sequence of two exons and a flanking region in caprine GDF9 gene, reporting the presence of polymorphism in GDF9 of four goat breeds, and the association between this polymorphism of GDF9 gene with litter size in goats of the Jining Grey breed. The polymorphisms scanning and typing of GDF9 gene uncovered four potentially meaningful SNPs in four goat breeds, in which the mutation G3288A was uncovered (Kasiriyan et al. 2011). In moderate prolificacy (Boer goat) and low prolificacy (Liaoning Cashmere goat) breeds, a linkage analysis indicated that there was more fervent linkage disequilibrium among loci 3288, 423 and 1189 compared to that detected in high prolificacy goat breeds of Jining Grey and Guizhou White (Feng et al. 2011). Actually, the three loci in Boer goats showed a clear linkage disequilibrium. All the above documented results provided enough evidence that the GDF9 gene structure is determinative in reproduction.

Another study by Eghbalsaied et al. 2014, performed on Iranian sheep, showed that G1-GDF9 mutation (arginine to histidine shift) occurred in Afshari breed, while G4-GDF9 mutation (glutamic to lysine shift) was detected in both Afshari and Shal breeds. A recent study in 2014, by Ahlawat et al., attempted to correlate six novel SNPs in BMPR1B, BMP15 and GDF9 genes to reproductive traits in Black Bengal goats.

The hypothesis that optimization of PCR parameters for each of specific sites on four genes within the Growth Differential Factor 9 (GDF9) might be different and complex has to be explored. Previous works related to optimization of PCR for different immunity and production genes of small ruminants showed important differences in the optimized parameters (Barbour et al. 2012). Other researchers pointed at the importance of PCR optimization of different sites on genes of the same animal specie that result in differences of the protocols' parameters (Vaiman et al. 1996; Ahlawat et al. 2014). Solving the complex optimization will help future researchers to adopt the optimized protocols, facilitating their progress to other objectives related to identification of genotypes in GDF9, and investigating their role in prolificacy of selected female offsprings.

The objective of this research is to overcome the complexity of optimizing the PCR protocols, in an attempt to amplify specific sites, G3288A, G423A, A959A, and A959C, located on four respective genes, P3, P4, P5, and P6, within the GDF9 in local ewes and does, a gene known to express required factor for folliculogenesis.

MATERIALS AND METHODS

Blood collection

Four ml of blood were collected aseptically from the jugular vein of mature Awassi breed ewes and female Local-Baladi breed does. The blood was placed in an ice chest for immediate transportation to the Animal Technology Laboratory at the American University of Beirut.

Buffy coat treatment and storage

Each blood sample was centrifuged for 10 minutes at 2000 rpm. A volume of 800 µl of dimethyl sulfoxide (5mg of DMSO in 100 µl of blood plasma) was added over 200 µl of the buffy coat containing the white blood cells. The Sulfoxide-supplemented buffy coat samples were stored at a temperature of -20°C degrees.

DNA extraction from Buffy coat cells

Extraction of the DNA from the stored buffy coat cells was performed using the manufacturer's instructions of the QIAamp kit (QIAGENGmbh, D-40724 Hilden, Germany). Extracted DNA was quantified using Nanodrop 2000c UV-VIS Spectrophotometer (Thermo Fisher Scientific, 168 Third Avenue, Waltham, MA, USA 02451)

Optimization of PCR protocols for the four genes of GDF9

Various primers were used to amplify the targeted four sites of GDF9. The targeted GDF9 sites, primer sequences and expected amplicon lengths are presented in Table 1.The primers were purchased from MOLBIOL (TIB MOLBIOL GmbH, Eresburgstrasse 22-23, D-12103 Berlin, Germany). Eight different trials were needed to solve the complexity in standardization of the amplification of these four sites, as detailed in the below experimental designs.

1. First Standardization with two DNA concentrations

The first PCR procedure was conducted in an attempt to amplify the four respective specific sites on P3, P4, P5, and P6 genes of the GDF9. This first attempt was conducted following the protocol of Feng et al., 2010, including two DNA concentrations (30 and 60 ng/50 ul of PCR mixture). The respective primers' volume and concentration in the PCR mix were set at 4 µl and 5 pmol. The cycling conditions in this first standardization were: Initial denaturation at 95º for 5 min, 32 cycles of denaturation at 94ºC for 30 sec, annealing at 60ºC for 30 sec, and extension at 72ºC for 1 min. PCR reactions were performed using CFX96 Touch™ Real-Time PCR Detection System (BioRad, 1000 Alfred Nobel Drive, Hercules, California 94547, USA). The PCR was conducted on randomly chosen sample of ewe's blood and a randomly chosen sample of a doe's blood. Electrophoresis was accomplished on 2 % agarose to document the image of banded amplicons.

2. Second standardization varying the cycling conditions

The DNA concentrations were set constant at 60 ng, and the primers were kept at a fixed volume of 4 µl in the PCR mix. The amplified genes were the P3, P4, P5, P6 of a doe and a ewe.

The cycling conditions were according to the manufacturer's instructions of RedTaq Ready mix kit (Sigma-Aldrich, 3050 Spruce Street, St. Louis, MO 63103 USA), set at 30 cycles, in which each cycle was programmed at 94◦C for 1 min, 60◦C for 2 minutes, and 72◦C for 3 minutes.

3. Third standardization varying cycling conditions and primer proportion

The third PCR standardization was conducted with a protocol adopted from the manufacturer's instructions of RedTaq Ready mix kit (Sigma-Aldrich, 3050 Spruce Street, St. Louis, MO 63103 USA) and of the work of Feng et al, 2010. DNA was set at a fixed concentration of 60 ng. The primers volumes were variable, set at 2 µl, 4 µl, and 8 µl. The annealing temperature for amplification of the site on the P3 gene was changed to 62◦C, while that of P6 was fixed at 60◦C. The PCR of gene P3 was applied on the buffy coat cells of a ewe and a doe, while that of the P6 gene was applied on buffy coat cells of a doe only.

4. Fourth PCR standardization of goat P3 gene varying concentrations of DNA, primers, and cycling conditions

The fourth PCR standardization of a doe's P3 gene included different annealing temperatures (50ºC, 54ºC, 58ºC, 62ºC), adoption of manufacturer's instructions of the RedTaq Ready mix kit (Sigma-Aldrich, 3050 Spruce Street, St. Louis, MO 63103 USA) versus that of Feng et al 2010, inclusion of two volumes of the P3 primer (2 µl and 4 µl) of 5 pMol concentration, and two DNA concentrations of 30 and 60 ng.

5. The fifth PCR standardization of doe's P3, using freshly extracted DNA, and same parameters of the 4th standardization.

6. The sixth standardization protocol for P3 and P6 of a ewe and a doe with varying DNA concentrations

The sixth PCR protocol was conducted according to RedTaq Ready mix kit instructions (Sigma-Aldrich, 3050 Spruce Street, St. Louis, MO 63103 USA) varying the annealing temperature between 54oC and 60oC The ewe and doe DNA concentration varied between 30 and 120 ng, while the primer was 4 or 5 µl of 5pMol.

7. The seventh PCR standardization of specific sites on P3 and P6 genes of a ewe and a doe with re-amplification of the PCR mix

The seventh PCR standardization of ewe's P3 and doe's and ewe's P6 genes involved the re-amplification of their products that were obtained in the 6th PCR standardization, , using two microliters of their previously amplified DNA, according to the conditions of the 6th protocol.

9. The eighth PCR standardization of a doe's P6 with varying cycling conditions

The eighth PCR standardization procedure was for a doe's P6. Two microliters of the previously amplified amplicon of the mix obtained from the 7th protocol were used for another cycles of amplification, using the following conditions: initial denaturation at 94◦C for 15 minutes, followed by 30 cycles of denaturation at 94◦C for 1 min, annealing at 60◦C for 1 min, extension at 72◦C for 1 min, and a final extension step at 72◦C for 7 min.

RESULTS & DISCUSSION

It is imperative to standardize and optimize PCR protocols that are to be used for repetitive polymorphic typing of specific genes involved in physiology of different animal systems. The complex PCR optimization is very time consuming and repetitive, due to the multitude of variables involved. Variables in PCR optimization include DNA template concentration and freshness, primer's concentration and design, Magnesium ion concentration (Innis et al. 1990), dNTPs concentration, PCR buffer systems, DNA polymerase concentration and selection, thermal cycling conditions, PCR additives and co-solvents. All these parameters, either individually or interdependently affect the outcome of optimized PCR protocol (Grunenwald 2003).

1. *First Standardization with two DNA concentrations*

Fig.1 showed the banded amplicons resulting from first PCR standardization with 30 ng DNA concentrations of a doe and a ewe, while Fig. 2 showed the banded amplicons resulting from first PCR standardization with 60 ng DNA concentrations of a doe and a ewe. Lanes 2, 7, 8, and 9 of Fig. 2 had clear amplicons corresponding to successful amplifications of respective genes P4 of a doe, P4 of ewe, P5 of ewe, and P6 of ewe, at a DNA substrate concentration of 60 ng. In Fig. 1, the Ladder was clearly banded in Lane 1, and the negative Lane 2 had absence of bands.

2. Second standardization varying the cycling conditions

Fig. 3 showed the banded amplicons resulting from the second PCR standardization, including 60 ng DNA concentrations of a doe, and setting conditions at 30 cycles of 94oC for 1 min, 60◦C for 2 minutes, and 72◦C for 3 minutes. Lanes 4-6 had clear amplicons corresponding respectively to P4, P5, and P6 of a doe.

Fig. 4 showed also the banded amplicons resulting from the second PCR standardization with 60 ng DNA concentrations of a ewe, and same cycling conditions used for the doe in Fig. 3. Lanes 4 and 5 showed clear amplicons corresponding respectively to P4 and P5 of a ewe. Results of Figs. 3 and 4, showed the need for a third standardization, targeting a solution for the amplification of the P3 and P6.

3. Third standardization involving variation in cycling conditions and primers volume

The amplification of P3, resulting from the third PCR standardization with 60 ng DNA concentration of a doe, and a ewe, and variable primer's volume of 2, 4, and 8 ul revealed no amplicon on electrophoresis. However, the banding of P6 amplicons, resulting from the same PCR standardization with 60 ng DNA concentration of a doe, 8 µl of primer, and adopting the protocol of Feng et. al 2010, including an initial denaturation temperature of 95◦C for 5 min, followed by 32 cycles of denaturation at 94◦C for 30s, and an annealing at 59◦C for 30 s, and an extension at 72◦C for 45 s, revealed a weak intensity-band in Lane 4 of Fig. 5. The failure in the third standardization procedure for amplification of the specific sites on both the P3 and P6 genes required further standardization. The following reagents are usually applied on the DNA sample subjected to a PCR procedure namely, template DNA (1-550 ng), primers (0.1-1µM), Mg2+ (0.5-5 mM), dNTP (20-200 µM each) (Innis et al. 1990; Ohler and Rose 1992) and DNA polymerase (0.5-2.5 U for each 50µl of a PCR reaction).

4. Fourth PCR standardization of a doe's P3 gene varying concentrations of DNA, primers volume, and cycling conditions

*T*he banding of a doe's P3 amplicon, resulting from the fourth PCR standardization with various DNA concentrations, primer volumes, and annealing temperature was attemped. The PCR conditions for amplicons loaded on specific lanes were adopted from Feng et al., 2010 (95◦C for 5 min, followed by 32 cycles of denaturation at 94◦C for 30s

Table 1: Sequences of Primers, designated target sites on the four genes (P3, P4, P5, P6) of GDF9, and expected amplicon size

Primer[1]	Targeted gene sites	Primer sequence	Expected amplicon size (bp)
P3F P3R	G3288A	CCTGTTACATATGGCATTAC TGTTGGAAT TTATCACCAGGTTGCATATA C	187
P4F P4R	G423A	GAACCTTTCCATCAGTGGATCAGCT AGCTCTAGGGAGAGTCTTGCT	182
P5F P5R	A959A	AAGCTGAGGGTGTAAGATC CATGGAGCTCACATTCATTC TG	150
P6F P6R	A959C	CCACACAAATACAACCCTCG ATAC AGGCTCGATGGCCAAAACACT	183

[1]The primers are given names of the targeted 4 genes namely, the P3, P4, P5, and P6. The 'F' stands for forward and the 'R' for reverse.

Table 2: Summary of the PCR standardization protocols for P3, P4, P5 and P6 of ewe and doe

				Cycling conditions					
Target gene	Species	Primer (µl)	DNA (ng)	Initial denaturation	Denaturing	Annealing	Extension	Amplification[a] Cycles	Agarose %
P3	Ewe	4	60	95◦C for 5 min	94°C 30 sec	58°C 30 sec	72°C 45 s	32 cycles	1
P3	Doe	4	60	95◦C for 5 min	94°C 30 sec	58°C 30 sec	72°C 45 s	32 cycles	2
P4	Doe Ewe	4	60	-	94°C 1min	58°C 2min	72°C 3min.	30 cycles	2
P5	Doe Ewe	4	60	-	94°C 1min	60°C 2min	72°C 3 min.	30 cycles	2
P6	Doe	4	120	-	94°C 1min	60°C 1min	72°C 1 min.	30 cycles	2
P6	Ewe	4	120	-	94°C 1min	60°C 2min	72°C 3 min.	30 cycles	1

[a] P3 of ewe, P6 of a doe, and P6 of a ewe are re-amplified for two, two, and three times, respectively

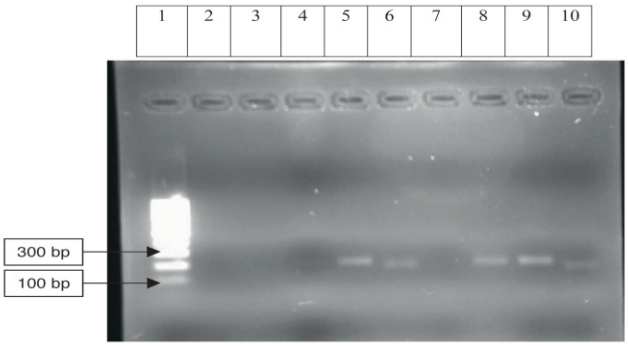

Figure 1: Banded amplicons resulting from the first PCR standardization with 30 ng DNA concentration of a doe and a ewe. Primer added in 4 µl volume. Lane 1, 100 bp Ladder; Lane 2, blank with no template DNA; Lanes 3, 4, 5, and 6 correspond to respective amplicons of doe's P3, P4, P5, and P6; Lanes 7, 8, 9, and 10 correspond to respective amplicons of ewe's P3. P4, P5, and P6. The cycling conditions in this first standardization were: Initial denaturation at 95ºC for 5 min, 32 cycles of denaturation at 94ºC for 30 sec, annealing at 60ºC for 30 sec, and extension at 72ºC for 1 min.

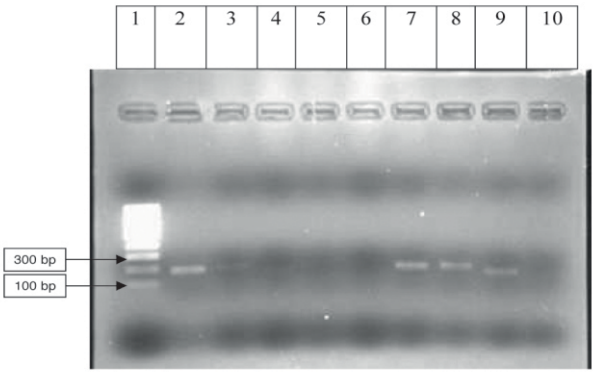

Figure 2: Banded amplicon resulting from the first PCR standardization with 60 ng DNA concentration of a doe and ewes. Primer added in 5 µl volume. Lane 1, 100 bp ladder; Lanes 2, 3, 4, and 5 correspond to respective amplicons of doe's P4, P5, P6, and P3; Lanes 6, 7, 8, and 9 correspond to ewe's P3, P4, P5, and P6; Lane 10, blank with no template DNA. The cycling conditions in this first standardization were: Initial denaturation at 95ºC for 5 min, 32 cycles of denaturation at 94ºC for 30 sec, annealing at 60°C for 30 sec, and extension at 72°C for 1 min.

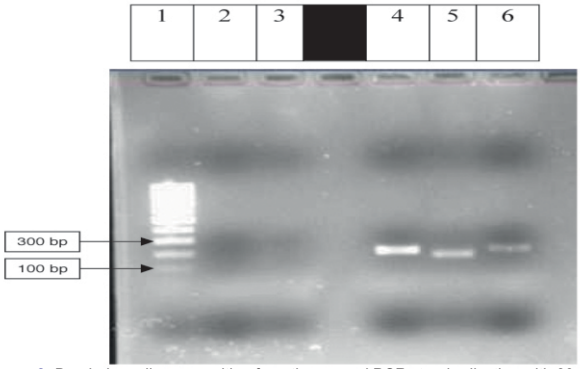

Figure 3: Banded amplicons resulting from the second PCR standardization with 60 ng of doe's DNA concentration, and 4 µl of primer, under conditions of 30 cycles at 94oC for 1 min, 60◦C for 2 min and 72◦C for 3 min; Lane 1, 100 bp ladder; Lane 2, blank with no DNA template; Lanes 3, 4, 5, and 6 correspond to respective doe's amplicons P3, P4, P5, and P6.

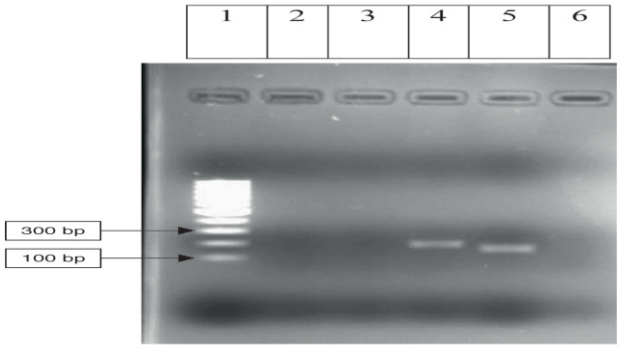

Figure 4: Banded amplicons resulting from the second PCR standardization with 60 ng of ewe's DNA concentration, and 4 µl of primer, under conditions of 30 cycles at 94oC for 1 min, 60◦C for 2 min and 72◦C for 3 min. Lane 1, 100 bp ladder; Lane 2, blank with no DNA template; Lanes 3, 4, 5, and 6 correspond to respective ewe's amplicons of P3, P4, P5, and P6.

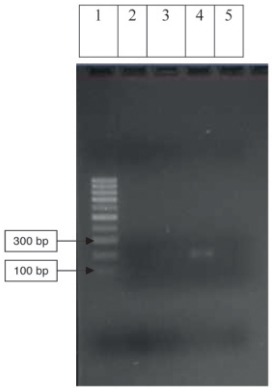

Figure 5: Banded P6 amplicons resulting from the third PCR standardization with 60 ng of doe's DNA and variable primers volumes. Lane 1, 100 bp ladder; Lane 2, a blank with no P6 primer; Lanes 3, 4, and 5 correspond to P6 amplicon generated at respective primers' volumes of 2, 4, and 8 µl; PCR conditions were adopted from the protocol of Feng et. al 2010, including an initial denaturation temperature of 95˚C for 5 min, followed by 32 cycles of denaturation at 94˚C for 30s, and an annealing at 59˚C for 30 s, and an extension at 72˚C for 45 s.

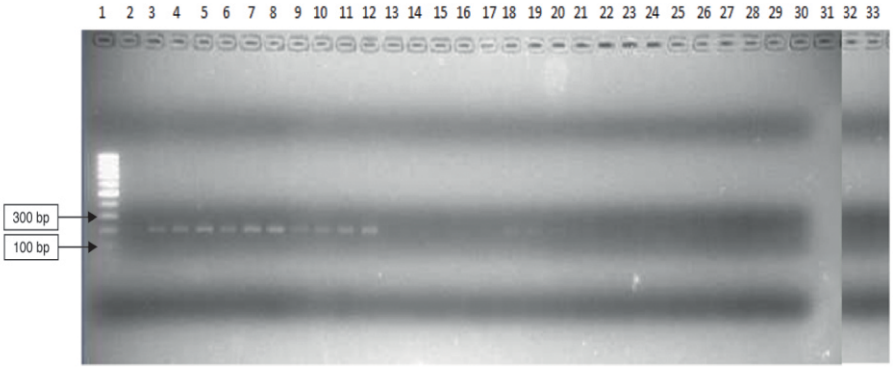

Figure 6: Banded doe's P3 amplicons resulting from the fifth PCR standardization with various concentrations of freshly extracted-DNA, different primer volumes and annealing temperatures (at). Lane 1, 100 bp ladder; Lanes with odd and even numericals used 2 and 4 µl of the primers, respectively. DNA level in each of Lanes 2,3,6,7,10,11,14,15,18,19,22,23,26,27,30, 31 was 30 ng, while each of the Lanes 4, 5, 8, 9, 12, 13, 16, 17, 20, 21, 24, 25, 28, 29, 32, 33 had 60 ng. The annealing Temp (at) was 50o C in Lanes 2, 3, 4, 5, 18, 19, 20, 21; at = 54oC in Lanes 6, 7, 8, 9, 22, 23, 24, 25; at= 58oC in Lanes 10, 11, 12, 13, 26, 27, 28, 29; at = 62°C in Lanes 14, 15, 16, 17, 30, 31, 32, 33. Cyclic conditions of Lanes 2 to 17 were according to RedTaq Ready mix kit, while cycling conditions of lanes 18-33 were adopted from Feng et al (2010).

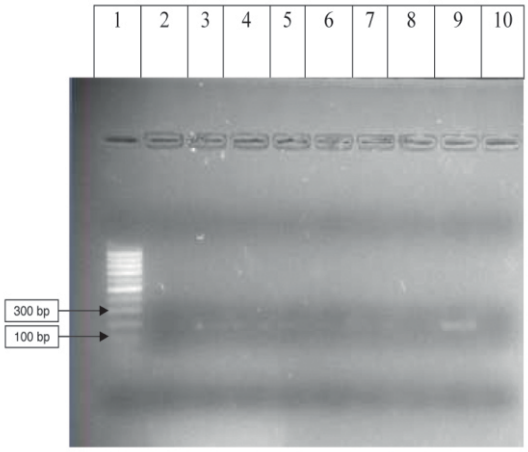

Figure 7: Banded P3 and P6 amplicons resulting from the sixth PCR standardization with various concentrations of freshly extracted-DNA, different annealing temperature (at) and different volumes of primers for P6 and P3. Lane 1, 100 bp ladder; Lane 8, blank with no DNA; The doe's DNA levels in Lanes 2, 3, and 4 and that of ewe's in Lanes 5, 6, and 7 were 30, 60, and 70 ng, respectively; the P6 was amplified in Lanes 2-4 for doe and 5 to 7 for ewe, while the ewe's P3 was amplified in Lanes 9 (30 ng of DNA) and 10 (60 ng of DNA). The primers volumes used in Lanes 9 and 10 were 2 and 4 µl, respectively. The varied applied annealing Temp (at) was: at = 60°C for Lanes 2, 3, 4, 5, 6, and 7; at = 54oC for Lanes 9 and 10. The PCR cyclic conditions were according to RedTaq Ready mix kit.

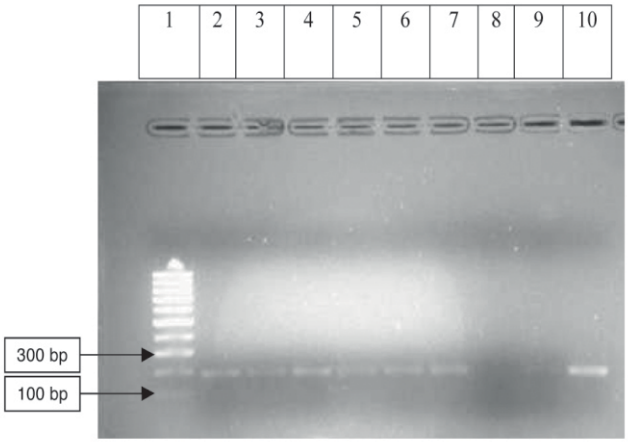

Figure 8: Re-amplification of previous ewe's P3 and doe's and ewe's P6 amplicons produced by the 6th PCR protocol , while adopting RedTaq Ready mix kit instructions, and banding products on 2% agarose gel, Lane 1, 100 bp ladder; Lane 8, blank with no DNA; Lanes 2, 3, and 4 had doe's P6 re-amplification of 2 µl of previous PCR run on respective DNA concentrations of 30, 60, and 120 ng; Lanes 5, 6, and 7 had ewe's P6 re-amplification of 2 µl of previous PCR run on respective DNA concentrations of 30, 60, and 120 ng; Lanes 9 and 10 had ewe's P3 re-amplification of 2 µl of previous PCR run on respective DNA concentrations of 30 and 60 ng.

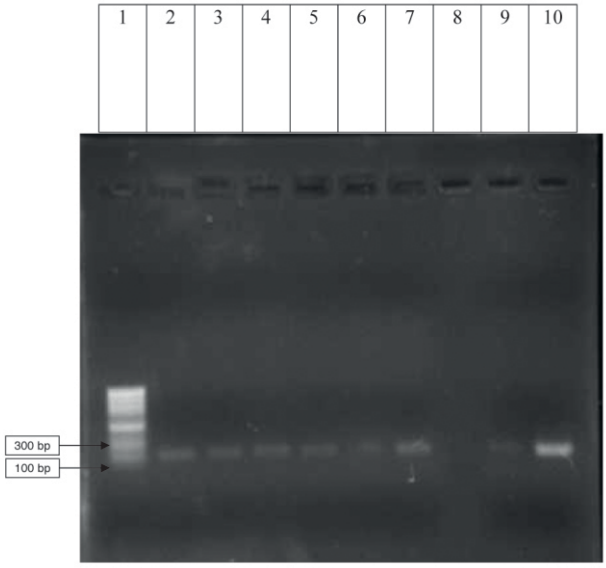

Figure 9: Banding the re-amplified amplicons produced in PCR of Fig. 8 on 1% agarose. Lane 1, 100 bp ladder; Lane 8, blank with no DNA; Lane 2, 3, and 4 had doe's P6 re-amplification of 2 µl of previous PCR run on respective DNA concentrations of 30, 60, and 120 ng; Lanes 5, 6, and 7 had ewe's P6 re-amplification of 2 µl of previous PCR run on respective DNA concentrations of 30, 60, and 120 ng; Lanes 9 and 10 had ewe's P3 re-amplification of 2 µl of previous PCR run on respective DNA concentrations of 30 and 60 ng.

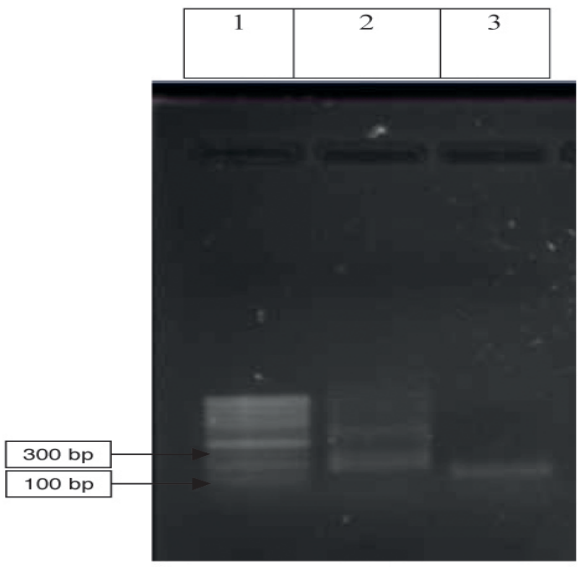

Figure 10: Banding the re-amplified amplicons of doe's P6 produced in PCR of Fig. 8, re-amplified and presented in Fig. 9, and re-amplified for a third time and banded in this figure on 1% agarose. The procedure adopted the instructions of the RedTaq Ready mix kit. Lane 1, 100 bp Ladder; Lane 2, amplicon generated by 40 PCR cycles; Lane 3, cycling conditions consisting of 30 cycles, annealing for 1min, extension for 1 min, and denaturation for 1 min

annealing temperature gradient from 50 to 62◦C for 30s, and extension at 72◦C for 45s), while the PCR conditions for other loaded lanes were adopted from the manufacturer's instructions of the RedTaq Ready mix kit (Sigma-Aldrich, 3050 Spruce Street, St. Louis, MO 63103 USA) (30 cycles of denaturation at 94◦C for 1 min, annealing at 62◦C for 2 min, and extension at 72◦C for 3 min). There was an absence of amplicons resulting from all the varied PCR mixtures and annealing temperatures.
The P3 and P6 amplifications suffered in failure through Standardizations 1 to 4, which was solved by introducing to the PCR mix a freshly extracted DNA (Standardization 5).

5. The fifth PCR standardization of a doe's P3 using freshly extracted DNA and same parameters of the 4th standardization

Fig 6 attempted the banding of a doe's P3 amplicons resulting from fifth PCR standardization with various concentrations of freshly extracted DNA, different primer volumes and annealing temperatures (at), similar to those used in the fourth PCR standardization. The PCR cyclic conditions of Lanes 1 to 16 were according to the manufacturer's instructions of the RedTaq Ready mix kit (Sigma-Aldrich, 3050 Spruce Street, St. Louis, MO 63103 USA), while the PCR cycling conditions of lanes 17-32 were adopted from Feng et al. (2010). Lanes 2 to 11 showed clear bands of doe's P3 amplicons, with differences in their intensities, while the other lanes had absence or faded amplicon bands.

In standardization 5, success was exhibited in a doe's P3 amplification, using primer concentration of 1µM, and following the protocol described by the PCR kit manufacturer (Sigma-Aldrich, 3050 Spruce Street, St. Louis, MO 63103 USA), but with lower annealing temperature of 58oC for a period of 30 s. Grunenwald (2003) stated that it was appropriate for PCR programs to start with an initial dissociation step between 92-95°C for 5 min, to ensure the complete separation of DNA strands. Furthermore, the length of the dissociation steps depend on the length and sequence of the targeted DNA strands. However, this research provided positive results, using the protocol provided by the manufacturer, without the inclusion of an initial dissociation step recommended by Grunenwald in the year 2003.

It is documented that Taq DNA polymerase would extend more than 60 nucleotides per second at a temperature of 70°C, while at a temperature of 75oC to 80°C the extension rate could reach up to 150 nucleotides per second (Gelfand 1989). Based on this fact, it is suggested that a final extension step be added for a period of 5-10 minutes at the temperature of 72°C. Longer annealing time between 20 s – 1 min, helped in having partial successful amplification in the 2nd to the 8th Standardizations used in this research; these results were in agreement with other documented research related to other genes (Barbour et al. 2012).

6. The sixth standardization of P3 and P6 including different DNA concentrations

Fig. 7 had the banding of P6 amplicons of a doe and a ewe, and the P3 for ewe only, resulting from the sixth PCR standardization, with various concentrations of freshly extracted-DNA, ranging between 30 to 120 ng per 50 microliters of the PCR mixture, and primer's volume of 4 or 5 µl. The PCR cyclic conditions were according to the manufacturer's instructions of the RedTaq Ready mix kit (Sigma-Aldrich, 3050 Spruce Street, St. Louis, MO 63103 USA) with an annealing temperature of 54oC or 60oC. Only Lane 10 showed a clear band of a ewe's P3 amplicon, using a DNA concentration of 60ng, with an annealing temperature of 54oC. Lane 9 had a low intensity-band of a ewe's P3, while the rest of lanes had an absence of bands. This result required further optimization of PCR for P3 and P6 genes in ewes and does.

The PCR failures exhibited in the 6th Standardization were initially believed to be due to inadequacies in primer and DNA template concentrations. It was later uncovered that the failure was due to the exhaustion of the Taq DNA Polymerase. This is reflected by the success of re-amplifications of the previous PCR products rather than being a problem in number of cycles; actually, standardizations 7 and 8 and their related Figs. 9 and 10 confirmed this reasoning.

7. The seventh PCR standardization protocol for re-amplification of ewe's P3 and doe's and ewe's P6 products obtained by the 6th protocol

Fig. 8 attempted the banding of P3 and P6 amplicons on 2 % agarose gel. The bands were the output of re-amplification of 2 µl of previous amplified DNA mix from the sixth protocol, using the same instructions of the RedTaq Ready mix kit (Sigma-Aldrich, 3050 Spruce Street, St. Louis, MO 63103 USA). Fig. 8 showed low intensity bands for P6 of a doe (Lanes 2-4), for P6 of a ewe (lanes 5-7) and for P3 of a ewe (lanes 9, and 10).

It was decided to attempt increasing the bands intensities, by another re-amplification of the PCR products obtained from the 7th protocol and banding on 1% agarose gel instead of the 2% gel. Fig 9 showed an improvement in obtaining more intense bands on the 1 % compared to the 2 % agarose gel. According to the results shown on Figs. 8 and 9, the P3 and P6 standardization, using the ewe blood cells, is found satisfactory; However, the standardization of P6 of a doe's buffy coat could be improved by other trials.

9. The eighth PCR standardization of a doe's P6 by re-amplification, varying cycling conditions, and banding on lower Agarose concentration

The banding of the amplicons of a doe's P6, by re-amplification of the PCR product banded in Fig. 8, re-amplified and banded in Fig. 9, and re-amplified for a third time and banded on 1% agarose (Fig. 10). The procedure adopted the manufacturer's instructions of the RedTaq Ready mix kit (Sigma-Aldrich, 3050 Spruce Street, St. Louis, MO 63103 USA. Fig. 10 showed a denaturation of the genomic copies in Lane 2, resulting in different base pair bands, possibly due to the application of 40 PCR cycles. However, Lane 3, resulting from 30 cycles, had an acceptable band of the doe's P6.

It is worth noting that the presence of a smear of different base pair-bands in lane 2 of Fig. 10 was due to the implementation of unacceptable number of 40 cycles (Grunenwald 2003). Furthermore, it should be noted that when the primer concentration was too high, the PCR amplification of P6 was negatively affected. Grunenwald (2003) and Innis et al. (1990) confirmed the hypothesis that PCR primer concentration could affect both the specificity and efficiency of the applied procedure. Usually, nonspecific priming and dimer formation occurs when the primer concentrations are high.

The Taq DNA polymerase has a half-life of 40 minutes at 95°C, rendering a sufficiency in polymerase activity during the first 30 cycles; thus, It is inadequate to include too many cycles in PCR amplification procedures. Amplification rates will reach to a plateau phase after a certain amount of the amplicon is synthesized. The limiting nature of DNA polymerase requires adoption of PCR amplification cycles between 20-40 cycles (Grunenwald 2003).

The solved complexity of PCR optimization for the four specific sites on their respective P3, P4, P5 and P6 genes of a ewe and a doe are summarized in Table 2.

CONCLUSION

In conclusion, the complexity in optimization of PCR protocols for the amplification of specific sites on four GDF9 genes of a ewe and a doe was overcame by a series of standardization trials, including changes in the DNA concentration, varying primer volumes, changing cycling conditions, adopting freshly extracted DNA, re-amplification of the PCR product, and varying concentrations of the agarose gel for banding the amplicons. The summary of the optimized protocols is shown in Table 2, which will help in paving the way for researchers to further elucidate the link between the polymorphic types of the sites present on the four gdf9 genes and prolificacy, which would in turn help in developing selection strategies for newly born female kids and lambs that will mature into respective reproductive does and ewes.

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