Animal Science Papers and Reports vol. 32 (2014) no. 3, 229-237
Institute of Genetics and Animal Breeding, Jastrzębiec, Poland

Development of cost-effective tetra-ARMS PCR for detection of FecB genotype in sheep*

Feng Guan[1], Guoqing Shi[2], Pengcheng Wan[2], Rong Dai[2], Hong Tang[2], Haixia Wang[1], Yuanyuan Luo[1]**

[1] College of Life Sciences, China Jiliang University, Zhejiang Provincial Key Laboratory of Biometrology and Inspection & Quarantine, Institute of Plant Environmental Biology and Measurement Technology, Hangzhou 310018, China

[2] The Key Sheep Breeding and Reproduction Biotechnology Laboratory of Xinjiang Production and Construction Group, Research Institute of Animal Husbandry and Veterinary, Xinjiang Agricultural Reclamation Academy, Shihezi 832000, China

(Accepted May 29,2014)

The FecB gene has been shown to be crucial in reproduction in many sheep breeds. It is a single nucleotide polymorphism (SNP) located in the bone morphogenetic protein receptor IB (BMPR-IB) gene. The current methods for genotyping the FecB mutation are either slow and laborious or expensive. In this report, a single-step amplification approach suitable for FecB genotyping method is described. Multiplex PCR was performed with four primers on the basis of tetra-primer amplification refractory mutation system PCR (tetra-primer ARMS PCR), then three FecB genotypes can be detected after electrophoresis. Genotyping results of the proposed multiplex PCR occurred to be in complete accordance with forced PCR-RFLP of all samples. It is a rapid and simple method for detection of FecB in a larger number of samples, and is suitable for other SNPs detection with specific primers even in most "low-tech" laboratories.

KEY WORDS: rapid detection / sheep FecB gene / tetra-ARMS PCR

Guoqing Shi and Feng Guan contributed equally to this work.

*Supported by the National Natural Science Foundation of China (No. 31360540), the National 863 Program (No. 2011AA100307), National Science and Technology Pillar Programe during the 12th Five-Year Plan of China (2011BAD28B05-1-1), Key Programs for Science and Technology Development of Xinjiang Production and Construction Group (2011BA006), the Ministry of science and technology state-level Spark Program (2012GA891001), and the National technological system of wool and cashmere industry (CARS-40-07).

**Corresponding author: yyluo@cjlu.edu.cn

The profitability of sheep farming mainly depends on lamb production per ewe and litter size. Both are important economical traits in sheep breeding and genetics, which mainly depend on breed. Different sheep breeds show variation in litter size across the world, and most deliver only a single lamb per lambing, while some produce twins or even triplets. There are a few sheep strains belonging to prolific breeds, such as Australian Booroola Merino, Chinese Hu, little tailed Han sheep, and others. Litter size is grossly dependent on ovulation rate which is under majority genetic control of a single gene [Davis *et al*. 2002, 2005, Roy *et al*. 2011]. Among the fecundity genes, the Booroola fecundity (FecB) gene was first identified in the Booroola Merino sheep in 1985 and described to affect ovulation rate and prolificacy in sheep. Later, it was proven that the FecB mutation was a point mutation in the coding region of the bone morphogenetic protein receptor IB (BMPR-IB) *locus* [Mulsant *et al*. 2001], and it has been found in many prolific breeds besides the Booroola Merino [Davis *et al*. 2002, Guan *et al*. 2007, Roy *et al*. 2011, Abdoli *et al*. 2013]. The mutation is non-conservative and is characterized by substitution of nucleotide adenine by guanine at 746 position of cDNA (A746G) resulting in substitution of amino acid arginine in place of glutamine at position 249 in the domain of the BMPR-IB gene. The FecB gene is additive for ovulation rate in ewes with the mutant allele [Mulsant *et al*. 2001, Souza *et al*. 2001], and in some reports it has been implicated in lower birth weight and slower post-weaning growth rate in lambs as well as a lighter mature body weight in ewes [Gootwine *et al*. 2006]. A positive effect has been shown on postnatal body growth in Chinese Merino prolific strain [Guan *et al*. 2007]. Due to these effects, the FecB gene has been widely screened across the world and introgressed into many non-carriers of prolific sheep breed to take advantage of the increased prolificacy.

The forced PCR-RFLP method was widely used to analyse the FecB mutation since it was developed by Wilson *et al* [2001] and modified by Davis *et al.* [2002]. To date, it was commonly used to genotype this gene, but the method requires the use of *Ava*II restriction enzyme which is more expensive than all PCR reagents and requires incubation for a longer time. Other methods such as DNA sequencing or PCR-SSCP were used [Ganai *et al*. 2012, Abdoli *et al*. 2013], but the procedures have disadvantages concerning the accuracy, cost, resolution, labour and time-consumption. These disadvantages become prohibitive with increasing sample size.

The tetra-primer amplification refractory mutation system PCR (tetra-primer ARMS PCR or T-ARMS PCR), also known as PCR-confronting two-pair primers (PCR-CPP) or Biallelic-ARMS, which was derived from general PCR, and specially utilized for detection of known SNPs, offers the advantages of fast and cost-effective detection utilizing a single PCR reaction to detect both alleles, with no need for a restriction enzyme [Ye *et al*. 1992, 2001]. It combines the advantages of amplification refractory mutation system (ARMS) and tetra-primer PCR. It is rapid and simple, with high accuracy, low cost and other good characteristics for SNP detection. In particular, it can discriminate whether the allele is homo- or heterozygous. Furthermore, modified tetra-primer ARMS PCR methods can detect more than one SNP simultaneously in a

single PCR reaction, which promotes efficiency of detection, reduces detection cost and time consumption [Lajin *et al*. 2012, Zhang *et al*. 2013].

The aim of the present study was to develop a tetra-primer ARMS PCR method that could simultaneously detect three FecB genotypes in sheep without any special equipment, and reduce the enzyme cost and time consumption.

Material and methods

DNA samples and protocol

DNA samples of a total of 100 sheep were used to develop the method. All DNA samples had been previously genotyped for FecB mutation by forced PCR-RFLP [Guan *et al*. 2007] as previously described by Wilson *et al*. [2001] and Davis *et al*. [2002] and some of the samples were direct sequenced. DNA samples included 6 Merino sheep (++) imported from Germany, 16 Merino×Hu crossbreds (+B) and 78 Hu (BB) sheep. The genomic DNA from ear skin tissue was extracted with phenol-chloroform and diluted with ddH_2O, of which 50 μL was aliquoted and stored at -80°C as a backup. The latter was used in this method.

Taq DNA polymerase, dNTPs, agents of PAGE electrophoresis and others were bought from Shanghai Sangon Biotechnology Co. Ltd.

Specific primers design

The tetra-primer ARMS PCR method was used to detect FecB mutation and employs four primers to amplify a larger fragment from genomic DNA containing the A746G mutation site and amplicons representing each of the two allelic forms. The specific primers were designed according to the published sequence of FecB gene (GenBank AF357007) using Primer Premier 5.0 software and web-based software made by Ye *et al.* [2001] (http://cedar.genetics.soton.ac.uk/public html/primer1.html) based on the principle of tetra-primer ARMS PCR, the specific primer sequences are listed in Table 1 and shown in Figure 1, termed primer F1 and R1, the bold type letter in pane represents A746G mutation *locus*.

Table 1. Primer sequences and protocol used in this study

Primer name		Sequences (5′ to 3′)	Primer concentration	Expected fragment
Control	F2	TCAGATGGTGAAACAGATTGGAAA	5 pM	220 bp
	R2	AGAAAGAGAGGAAAGCTTGGCTAAGC		
Specific	F1	GCTGGTTCCGAGAGACGAAAATATAGCG	20 pM	G allele 120 bp
	R1	GTTTTTGATGCCTCATCAACACCGGCT		A allele 160 bp

In the sequences of two specific primers, the base of downstream primer at 3′ terminus matches with ++ genotype DNA sequence but does not match with BB genotype sequence, the chain of individuals with BB genotype cannot extend because

Fig. 1. Specific primers used in this study are shown above the sequences. Marked letters indicate the deliberatly introduced mismatch nucleotide to enhance the 3′ mismatch specificity.

it is difficult to form the phosphodiester bond at 3′ terminal mismatched base, so individuals with BB genotype have no correspondingly specific PCR product because the PCR amplification reaction was stopped. Otherwise, the specific upstream primer matches with BB genotype DNA sequence but does not match with ++ genotype DNA sequence, the individuals with ++ genotype have no correspondingly specific product. The specific amplicons for BB and ++ genotypes have different lengths in the PAGE electrophoresis. For the heterozygous individuals, they have BB and ++ genotypes and have two specific fragments in the PCR amplification.

To enhance allelic specificity, a second deliberate mismatched base (indicated by frame in Fig. 1) was incorporated at position -3′ terminus in the specific primers according to the principle described by Ye *et al.* [2001].

Control primers design

A pair of primers was designed at the two flanks of mutational site A746G as control. To get the appropriate length fragment as control in the electrophoresis, the downstream control primer was designed in the nearest intron region, which was amplified and sequenced, located at 194 bp downstream of A746G and about 1 kb long in BMPR-IB gene. The control primers used are termed F2 and R2 and the sequences are listed in Table 1. The anticipated fragment length and primer concentration are given in Table 1.

All the primers in this study were synthesized in Shanghai Songon Bio-technical Company.

Optimization for tetra-primer ARMS PCR system

In addition to the specificity of primers in tetra-primer ARMS PCR method, the important procedure was optimization of the amplification system. In this study, the dosage of template DNA was about 50 ng, other amplification factors were optimized one by one with all other factors fixed, these factors included annealing temperature, concentrations of primers, Mg^{2+}, dNTP and dosage of *Taq* DNA polymerase.

In this study, annealing temperature at 5 different levels (64°C, 62°C, 60°C, 58°C and 56°C) was optimized while the conditions of other PCR factors were fixed. Under the condition of optimized annealing temperature, Mg^{2+} concentrations (1.5, 1.8, 2.0, 2.5, 3.0 mM), dNTP concentrations (0.15, 0.20, 0.25, 0.3 mM), the dosage of *Taq*

polymerase, and the cycles (25, 30, 35) of PCR were optimized. Under the optimized conditions, the sensitivity with different concentration ratios of specific and control primers (4:1, 2:1, 1:1, 1:2, 1:4) was optimized.

PCR amplification and electrophoresis

After optimization, PCR amplification was carried out in a total volume of 20 μL reaction system containing about 50 ng template DNA, 2μL 10X buffer, 1.5 mM $MgCL_2$, 0.2 mM of each dNTP, 1 U of *Taq* polymerase, 1μM of each specific primer, 0.25 μM of each control primer. Amplifications were carried out in an MJ (PTC100, USA) thermal cycler.

The thermal profile consisted of the following: initial denaturation at 95°C for 5min; followed by 30 cycles of denaturation at 94°C for 30 sec, annealing at 62°C for 30 sec, and extension at 72°C for 30 sec. The final extension step was at 72°C for 5 min. Eight microliters of PCR products was loaded on 10**%** PAGE gel which was run with 1×TBE buffer for 3.0 h under a constant voltage of 120 V at room temperature and visualized with silver staining.

Results and discussion

Optimization of tetra-primer ARMS PCR conditions

After the optimization of all reaction conditions in tetra-primer ARMS PCR, the optimized conditions for PCR amplification were determined on the basis of annealing temperature, PCR reagents and concentrations, which were used in PCR reaction system as above. The template DNA content of PCR reaction volume was about 50 ng according to the report by Ye *et al.* [2001]. Under the optimized conditions, the extension from 3′ terminal mismatched base was just blocked. That is to say, for the individuals with BB genotype, the PCR products have 220 bp control and 120 bp specific fragments. The individuals of ++ genotype have 220 bp control and 160 bp specific fragments in the PCR products. The sheep with this two homozygous genotypes had no other non-specific amplification fragment. The sheep with FecB heterozygous genotype had all the 220 bp control fragment, 120 bp and 160 bp specific fragments. There was no false positive and/or false negative amplification under these optimized conditions.

Comparison of the assays

The same DNA samples were analysed using our developed method and forced PCR-RFLP and sequenced. The PCR products and enzyme processed PCR products were separated on PAGE gel, respectively. The electrophoresis pattern of tetra-primer ARMS PCR (Fig. 2, left) is more clear than that of PCR-RFLP method (Fig. 2, right).

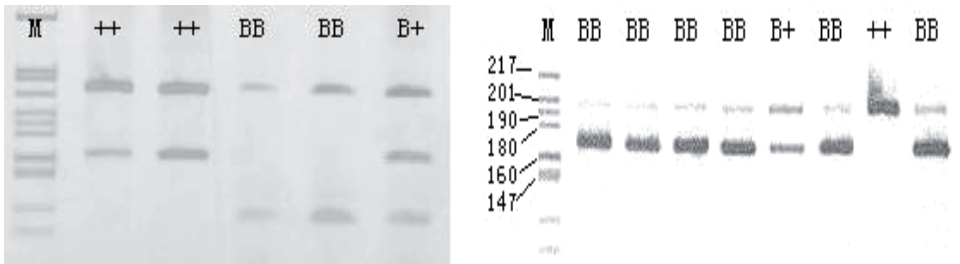

Fig. 2. Comparison of electrophoresis profiles between tetra-primer ARMS PCR (left figure) and PCR-RFLP (right figure) methods for detection of FecB gene in sheep.

The genotypes of 100 sheep DNA samples detected with tetra-primer ARMS PCR were in complete accordance with the results obtained by PCR-RFLP method and sequencing, Hu sheep all had the BB genotype, Merino only had the ++ genotype, and crossbred Merino×Hu sheep had the B+ genotype.

Comparing of methods in question, the tetra-primer ARMS PCR occured simpler than that of PCR-RFLP and sequencing. The developed method was cost effective and fast enough to allow testing of a large number of samples. Furthermore, tetra-primer ARMS PCR decreases time cost and saves incision enzyme, so it can minimize the effects of enzyme incision, especially from incomplete enzyme digestion.

Since tetra-primer ARMS PCR method was reported in detail by Ye *et al.* [2001], it has become one of the most commonly used methods for SNP genotyping. The principle of tetra-primer ARMS PCR is that *Taq* DNA polymerase lack of 3′→5′ exonuclease activity, so the extension rate at 3′ terminal mismatched primer is slower than normal 3′ terminal primer or cannot extend.

The tetra-primer ARMS PCR is used to detect a known SNP and employs four primers to amplify a larger fragment of DNA containing the mutation site and amplicons representing each of the two allelic forms. Primers can be designed to amplify different size fragments for each allele band in order to be easily resolved using electrophoresis. Two allele-specific primers are designed in opposite orientation and in combination with the control primers, which can simultaneously amplify both the wild type and the mutant amplicons [Ye *et al*. 2001]. According to the tetra-primer ARMS PCR principle, one reaction for a certain SNP produces two or three PCR products for homozygotes or heterozygotes, respectively. Furthermore, modified multiplex tetra-primer ARMS PCR can produce many more different length fragments in a single PCR reaction according to the number of SNP *loci* detected [Lajin *et al*. 2012, Zhang *et al*. 2013]. The tetra-primer ARMS PCR has many advantages over PCR-RFLP, real-time PCR and DNA sequencing in term of cost, time consumption and applicability in a typical laboratory [Etlik *et al*. 2011].

Several tetra-primer ARMS PCR-derived methods were reported for genotype inherited and acquired mutations in animals and humans, and even spinal muscular atrophy (SMA) gene deletion analysis [Baris *et al*. 2010]. These methods combined

with the advantages of tetra-primer ARMS PCR and multiplex PCR, PCR product assay, and developed novel tetra-primer ARMS PCR, which could genotype one more SNPs in a single PCR reaction simultaneously. It promotes detection efficiency and reduces cost, time consumption and workload. Lajin *et al.* [2012] and Zhang *et al.* [2013] reported developed multiplex tetra-primer ARMS PCR methods which could genotype three and six SNPs simultaneously, respectively. In a tube, a total of 24 primers were pooled to amplify 12 target alleles of 6 SNPs in a single PCR reaction with four different annealing temperatures. Results were identical to DNA sequencing [Zhang *et al.* 2013], this assay has a potential in SNP analysis especially for a larger number of samples and many SNPs.

The key procedure in tetra-primer ARMS PCR is the design of specific primers, which determines the sensitivity of analysis. The 3′ terminal base of specific primer must be located in the mutational base or it cannot judge whether the mutation is happened. The 3′ terminal base of specific primer can match with the DNA sequence of wild-type, but mismatch with the sequence of mutation type, or vice versa. The sensitivity must be optimized by PCR reaction conditions, such as the concentrations of specific and control primers, dosage of *Taq* polymerase and annealing temperature. Even more, to enhance allelic specificity, a second deliberate mismatch at position -1, -2 or -3 from the 3′-terminus was introduced in specific primers [Ye *et al*. 2001, Etlik *et al*. 2011, Lajin *et al*. 2012, Zhang *et al*. 2013]. In fact, introduction of a second deliberate mismatched base had also depended on types of mismatched bases. For example, mismatched types were divided into strong, weak and medium levels, only weak and medium mismatches require a second mismatched base at according position [Ye *et al*. 2001]. In this analysis, a second deliberate mismatched base was introduced into at position -3 of the two specific primers to increase the specificity, respectively. Furthermore, the primer concentrations affected the PCR sensitivity. Generally, the concentrations of primers and polymerase in a mismatched PCR amplification are lower than that of common PCR amplification [Yan *et al*. 2002, 2003]. In multiplex tetra-primer ARMS PCR, added betaine could improve the amplification of GC-rich templates with strong secondary structures, and facilitate method development and conditions optimization [Lajin *et al*. 2013].

Tetra-primers ARMS PCR was an effective assay for a known SNP, which can be combined with multiplex PCR, post-PCR products treatments and conditions optimization to develop a more effective assay. It is interesting that two or more SNPs can be analysed in a single PCR reaction simultaneously, which is simpler than other assays. Up to now, this method had been widely used in the detection of SNPs in human and animal studies, and it has wide potential and applications for SNP detection as well as deletion and insertion polymorphisms.

Acknowledgements. *We thank Sarah Fogenburg for English editing and comments.*

REFERENCES

1. ABDOLI R., ZAMANI P., DELJOU A., REZVAN H., 2013 – Association of BPR-1B and GDF9 genes polymorphisms and secondary protein structure changes with reproduction traits in Mehraban ewes. ***Gene*** 524(2), 296-303.
2. BARIS I., ETLIK O., KOKSAL V., ARICAN-BARIS S.T., 2010 – Rapid diagnosis of spinal muscular atrophy using tetra-primer ARMS PCR assay: simultaneous detection of SMN1 and SMN2 deletion. ***Molecular and Cellular Probes*** 24, 138-141.
3. DAVIS G.H., GALLOWAY S.M., ROSS L.K., GREGAN S.M., WARD G., NIMBKAR B.V., GHALSASI P.M, NIMBKAR C., GRAY G.D., INOUNU I., TIESNAMURTI B., MARTYNIUK E., EYTHORSDOTTIR E., MULSANT P., LECERF F., HANRAHAN J.P., BRADFORD G.E., WILSON T., 2002 – DNA tests in prolific sheep from eight countries provide new evidence on origin of the Booroola (FecB) mutation. ***Biology of Reproduction*** 66, 1869-1874.
4. DAVIS G.H., 2005 – Major genes affecting ovulation rate in sheep. ***Genetics Selection Evolution*** 37, S11-23.
5. ETLIK O., KOKSAL V., ARICAN-BARIS S.T., BARIS I., 2011 – Development and validation of a cost-effective in-house method, tetra-primer ARMS PCR assay, in genotyping of seven clinically important point mutations. ***Molecular and Cellular Probes*** 25, 177-181.
6. GANAI T.A., MISRA S.S., SHABIR M., 2012 – Polymorphism analysis of BMPR1B gene by forced RFLP and PCR-SSCP techniques and expression of the mutation in introgressed sheep. ***Tropical Animal Health and Production*** 44, 277-83.
7. GUAN F., LIU S.R., SHI G.Q., ANG L.G., 2007 – Polymorphism of FecB Gene in nine Sheep Breeds or Strains and Its Effects on Litter Size, Lamb Growth and Development. ***Animal Reproduction Science*** 99, 44-52.
8. GOOTWINE E., ROZOV A., BOR A., REICHER S., 2006 – Carrying the FecB (Booroola) mutation is associated with lower birth weight and slower post-weaning growth rate for lambs, as well as a lighter mature bodyweight for ewes. ***Reproduction Fertility and Development*** 18, 433-437.
9. LAJIN B., ALACHKAR A., SAKUR A.A., 2013 – Betaine significantly improves multiplex tetra-primer ARMS-PCR methods. ***Molecular Biotechnology*** 54, 977-982.
10. LAJIN B., ALACHKAR A., SAKUR A.A., 2012 – Triplex tetra-primer ARMS-PCR method for the simultaneous detection of MTHFR c.677C>T and c.1298A>C, and MTRR c.66A>G polymorphisms of the folate-homocysteine metabolic pathway. ***Molecular and Cellular Probes*** 26, 16-20.
11. MULSANT P., LECERF F., FABRE S., SCHIBLER L., MONGET P., LANNELUC I., PISSELET C., RIQUET J., MONNIAUX D., CALLEBAUT I., CRIBIU E., THIMONIER J., TEYSSIER J., BODIN L., COGNIÉ Y., CHITOUR N., ELSEN J.M., 2001 – Mutation in bone morphogenetic protein receptor-IB is associated with increased ovulation rate in Booroola Merino ewes. ***Proceedings of The National Academy of Sciences of the United States of America*** 98, 5104-5109.
12. ROY J., POLLEY S., DE S., MUKHERJEE A., BATABYAL S., PAN S., BRAHMA B., DATTA T,K., GOSWAMI S.L., 2011 – Polymorphism of fecundity genes (FecB, FecX, and FecG) in the Indian Bonpala sheep. ***Animal Biotechnology*** 22, 151-162.
13. SOUZA C.J., MACDOUGAL C., CAMPBELL B.K., MCNEILLY A.S., BAIRD D.T. 2001 – The Booroola (FecB) phenotype is associated with a mutation in the bone morphogenetic receptor type 1B (BMPR-1B) gene. ***Journal of Endocrinology*** 169, R1-6.
14. WILSON T., WU X., JUENGEL J.L., ROSS I.K., LUMSDEN J.M., LORD E.A., DODDS K.G., WALLING G.A., MCEWAN J.C., O'CONNELL A.R., MCNATTY K.P., MONTGOMERY G.W., 2001 – Highly prolific Booroola sheep have a mutation in the intracellular kinase domain of bone morphogenetic protein IB receptor (ALK-6) that is expressed in both oocytes and granuloma cells. ***Biology of Reproduction*** 64, 1225-1235.

15. YAN Z.Q., TONG Q., WANG F., WU P.C., YANG S.L., GONG Y., 2002 – Use of a rapid mismatch PCR method to detect gyrA and parC mutations in ciprofloxacin-resistant clinical isolates of Escherichia coli. ***Journal of Antimicrobial Chemotherapy*** 49, 549-552.
16. YAN Z.Q., YANG S.L., GONG Y., 2003 – Use of PCR related methods in detection of gene mutation. ***Hereditas*** (Beijing) 25, 198-200.
17. YE S., DHILLON S., KE X., COLLINS A.R., DAY I.N., 2001 – An efficient procedure for genotyping single nucleotide polymorphisms. ***Nucleic Acids Research*** 29, E88-8.
18. YE, S., HUMPHRIES S., GREEN F., 1992 – Allele specific amplification by tetra-primer PCR. ***Nucleic Acids Research*** 5,1152.
19. ZHANG C., LIU Y., RING B.Z., NIE K., YANG M., WANG M., SHEN H., WU X., MA X., 2013 – A Novel Multiplex Tetra-Primer ARMS-PCR for the Simultaneous Genotyping of Six Single Nucleotide Polymorphisms Associated with Female Cancers. ***PLoS ONE*** 8, e62126.