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Detection of polymorphism in BMPR-1B gene associated with litter size in Awassi sheep ewes using PCR-RFLP method

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Abstract

This study was conducted to investigate the multiple aspects of mutant fertile gene and their effects in reproductive performance of Iraqi Awassi ewes. A total of 82 Iraqi Awassi ewes (2-3) years old with an average live body weight (45kg) were obtained from two locations (First, College of Agriculture, University of Baghdad and second, Agricultural Research Station) in Abu-Ghraib, (30km) south- west of Baghdad and were used in this study from August 2009 to August 2010. Investigation was carried out for multiple fertility gene (BMPR-1B) using the (PCR-RFLP) technique. Blood samples (5cm/ewe) were collected from the jugular vein using vacutainer tubes with anticoagulant material (ACD). Genomic DNA was extracted from the whole blood for each sample using (Wizard genomic DNA purification Kit). The concentration and the purity of extracted DNA were measured using Spectrophotometry. Gel concentration (0.8%) was used for the purpose of electrophoresis. Specific parts of mutant region were isolated and amplified using (polymerase chain reaction) and specific (DNA markers). For the purpose of natural point mutation diagnosis in the resulting PCR product is through the digestion of (BMPR-1B) gene (140bp), by using restriction enzyme (*Ava II*).The results of the study showed that: Awassi ewes studied with (++) genotype revealed the absence of fertility gene (BMPR-1B). Twining percentage found in some Awassi ewes were not related to this gene and maybe are related to other fertility genes or to other factors such as nutrition. Further studied required to evaluate the relationship of different genotypes with litter size and ovulation rate.

Keywords: Polymorphism, BMPR-1B gene, PCR-RFLP, Litter size, Awassi sheep, Iraq.

Introduction

Iraqi sheep breeds are characterized by low reproductive efficiency and twining rates (Younis, 1977). Recent studies have reported that the high prolificacy in many prolific sheep breeds around the world is the result of the found FecB gene (Galloway *et al*., 2000; Davis, 2004. Newly developed DNA tests have encouraged researchers to screen for the presence of these mutations in many local sheep breeds around the world. High prolificacy in Booroola sheep is due to a non-conservative mutation in a highly conserved intracellular kinase signaling in bone morphogenetic protein receptor-1B (BMPR-1B) expressed in the ovary and granulosa cells (Mulsant *et al*., 2001 and Wilson *et al*., 2001). The BMPR-1B, is a member of the transforming growth factor-B (TGF-B) superfamily. These are multifunctional proteins that regulate growth and differentiation in many cell types. Fecundity genes have posed the unique and exciting opportunity to add a high level of prolificacy to sheep that fit the environment well, without having to add undesirable traits of another breed. These mutations can be detected directly by forced PCR restriction fragment length polymorphism (RFLP) approach based on the reports described by (Souza *et al*., 2001 and Davis *et al*., 2002). The objectives of this study were investigateD the presence of the (BMPR-1B) gene by PCR- restriction fragment length polymorphism in Awassi sheep ewes, evaluated the effects of the (BMPR-1B) gene on litter size and body weight after birth in the Awassi sheep ewes.

Materials and Methods

Location of the experiment: This study was conducted at two locations in Abu-Ghraib (30km south- west of Baghdad), the first location was at the Animal farm, College of Agriculture, University of Baghdad (Flock number one consisted of 30 Awassi ewes), while the second flock number two consisted of 52 Awassi ewes was located at Agricultural Research station ministry of the

Agriculture. A total of eighty two Iraqi Awassi ewes, (2-3) years old with an average live body weight (45kg) were used in this study from August 2009 to August 2010. At lambing ewes and lambs were identified with spray in addition to the plastic numbers. Litter size, body weight, type of brith, sex of lambs and other relevant information were recorded. Animals fed concentrate 2% of their body weight were divided into two halves. Roughage (green alfalfa, straw) was offered *ad libitum*, and the animals were allowed to graze natural pasture. Water was available at all times. All ewes were protected against foot and mouth disease (FMD), Enterotoxaemia and were drenched against endoparasites.

Blood collection and DNA extraction: Approximately, 5ml venous blood was collected from each ewe. Blood was placed in tubes containing anti-coagulant solution acid citrate dextrose (ACD) and stored at (20°C). Blood samples were used as a source of DNA for PCR-RFLP detection of BMPR-1B gene in Awassi sheep ewes. Genomic DNA Extraction from Frozen Blood (Promega DNA Wizard).

Detection of the BMPR-1B gene in Awassi sheep by PCR-RFLP: Polymerase chain reaction (PCR) was carried out using a modification of the forced restriction fragment length polymorphism (RFLP) method described by (Hanrahan *et al*., 2004). The primer reverse (5-CAAGATGTTTTCATGCCTCATCAAC-ACGGTC-3) and primer forward (5- GTCGCTATGGG-GAAGTTTGGATG-3) has been engineered to introduce a point mutation resulting in PCR products with BMPR1B mutation containing an *AvaII* restriction site (G/GACC), whereas products from non-carriers lack this site. The (140bp) product was digested by *AvaII* (Chu *et al*., 2006). Products containing the BMPR-1B mutation were digested to yield a (110bp) and (30bp) fragment, whereas non-carriers products remained uncut at (140bp).

PCR amplification of BMPR-1B gene: The PCR amplification was performed in a total volume of 25µl containing 2 µl DNA, 12.5 µl Go Taq green master mix 2X (Promega corporation, USA), 0.75µl of each primer (10 pmol) then the volume was completed with 25µl of nucleases free water. The thermal cycling conditions were done as follows: Denaturation at 94 °C for 5min, followed by 35 cycles of 94 °C for 30s, 60°C for 30s and 72 °C for 30s with final incubation at 72°C for 5 min using a thermal Cycler (Gene Amp, PCR system 9700; Applied Biosystem), then hold at 4°C. The PCR reactions were tested by horizontal 2.5% agarose gel electrophoresis.

PCR – RFLP for BMPR-1B gene: Restriction Enzyme *AvaII* (Promega, Madison, WI, USA) Digest Analysis: Restriction digests were performed on PCR products to identify if a mutation is present in a DNA samples. For PCR product digests. In a sterile tube, assemble in order:

Sterile D.W	11.55µl
RE 10X Buffer	2µl
Acetylated BSA, 10µg/µl	0,2µl
DNA, Mix by pipetting, then add:	5µl
Restriction Enzyme(*AvaII)*	1.25µl
Final volume	20µl

Mixed gently by pipetting, close the tube and centrifuge for a few seconds in a micro-centrifuge. The reactions were then incubated for at least 4 hours or overnight at the specified temperature for the enzyme (usually 37°C). After heat inactivation the product of the restriction digest, 2 to 5 µl of the digested sample was taken, added loading buffer, and were visualized by electrophoresis on 2.5% agarose gel volt and time of electrophoresis to check the result (promega Madison, U.S.A). One pocket per row was used for the length standard (100 DNA ladder), volume 3µl of length standard was used to estimate the size and concentration of the PCR products, the gel was visualized with ethidium bromine.

Genotype analysis: The forced PCR of the BMPR-1B gene produced a (140bp) band. The BMPR-1B gene homozygous carriers could be identified as having a (110bp) band (BB), the non-carriers as having a (140bp) band (++), whereas and the heterozygotes as having both the (110bp) and (140bp) bands.

Results and Discussion

Detection of the BMPR-1B mutation gene: In this study, total of (82) individuals of Awassi sheep were genotyped with the PCR-RFLP approach (Figure1). The results showed in this study that the frequency of polymorphism distributions of BMPR-1B mutation gene was imbalanced in breed (Table 1). All genotyped sheep had the wild type allele (++). One genotype, (++) (140bp/140bp) were detected in Awassi sheep (Figure 1) . Sequencing verified the presence (or absence) of the polymerphic *AvaII* cleavage site as assessed by agarose gel electrophoresis. The results of this study indicated that Awassi ewes non-carried the same BMPR-1B mutation gene as were found in Shal ewes (Iran) (Mokhtar *et al*., 2009).

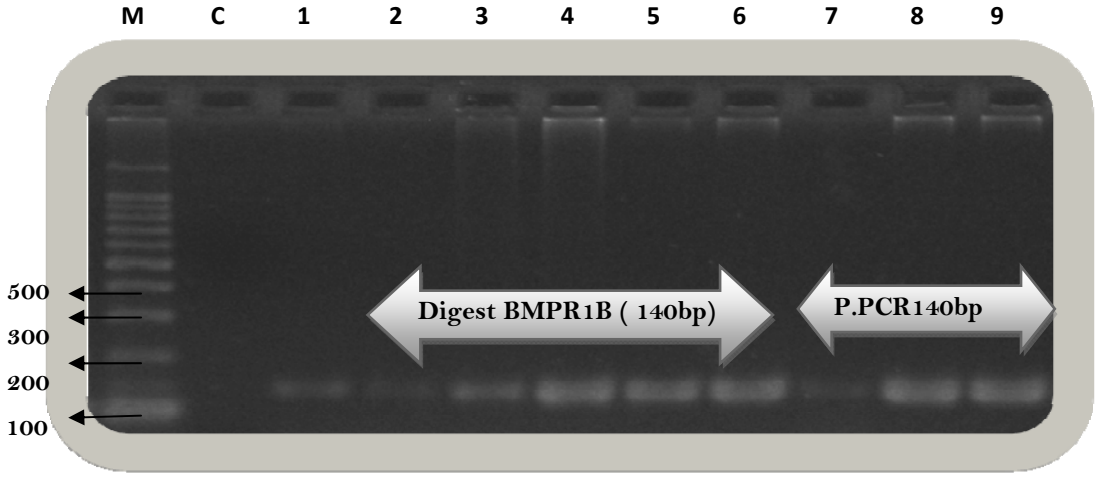

Figure (1): PCR amplification of BMPR-1B gene (140bp, Lanes 8-9) in Awassi sheep breed and digestion product of samples with *Ava II* restriction enzyme (lanes 1-6) with genotype (++) . Lane C, negative control and Lane M, molecular size marker (100bp DNA ladder).

Table (1): The frequency distributions of BMPR-1B gene in Awassi sheep .

Gene	Number of ewes	Allelic frequency		Genotypic frequency		
BMPR-1B	82	+	G	++	B+	BB
		1	0	1	0	0

The BMPR-1B mutation is present in Booroola Merino (Australia) (Mulsant *et al*., 2001; Souza *et al*., 2001; Wilson *et al*., 2001), Garole (India) (Davis *et al*., 2002), Javanese (Indonesia) (Davis *et al*., 2002), Small Tailed Han (China) (Liu *et al*., 2003; Wang *et al*., 2003a; Jia *et al*., 2005; Yan *et al*., 2005; Davis *et al*., 2006), and Hu-sheep (China) (Wang *et al*., 2003a,b, 2005; Yan *et al*., 2005; Davis *et al*., 2006; Guan *et al*., 2006; Al-Barzinji, 2010). In contrast to these findings the result of this study showed that the BMPR-1B mutation is not present in Awassi sheep (Iraqi). BMPR-1B mutation Booroola sheep have an increased ovulation rate and an average litter size of one to two extra lambs per copy of the BMPR-1B gene. Animals heterozygous and homozygous for the BMPR-1B gene are referred to as B+ and BB animals respectively. The mutation in BMPR-1B was identified as a point mutation in a highly conserved region of the intracellular kinase signalling domain (Wilson *et al*., 2001). Expression of mRNA encoding BMPR-1B was observed in oocytes from the type 1 stage of follicular development in both wild types (++) and (BB) genotypes. Granulosa cells did not express BMPR1B mRNA until the type 2 stage of follicular development in the (++) and (BB) genotypes. Oocytes in the small follicles of (BB) ewes were larger than in equivalent sized follicles of (++) animals. The oocytes reached a mean diameter of 130.5 µm in (BB) follicles with a mean diameter of 236µm (type 4 follicles) and in (++) follicles with a mean diameter of 383µm (type 5 follicles). Thereafter, the changes in oocyte diameter were similar for both genotypes follicles in (BB) ewes ovulate at a diameter of 3000µm with a mean number of 1.9 million granulosa cells in the pre-ovulatory follicles. In (++) ewes, the follicles ovulated when they are between 5000 and 6000µm in diameter with a mean number of 530 million granulosa cells (McNatty *et al*., 2005). Granulosa cells from (BB) ewes displayed a significantly higher FSH-induced cAMP responsiveness together with an earlier onset of follicular steroidogenesis and LH responsiveness than granulosa cells from (++) animals. The end result of these changes in the BMPR-1B mutated ewes was that maturation and ovulation of multiple ovarian follicles occured at smaller follicular diameters. It looks likely that the mutation in BMPR-1B prevents the normal inhibition of granulosa cell differentiation by a BMP (McNatty *et al*., 2005). Both the heterozygous BMP-15 and the BMPR-1B mutations caused changes to

the oocytes of early preantral follicles and resulted in more rapid follicular maturation and the ovulation of follicles with fewer granulosa cells. This is in contrast to the effect of the FecB mutation in sheep, which exhibited an earlier onset of aromatase activity and increases in both ovulation rate and litter size.

Conclusion

In the present study the results of PCR showed the same band pattern in all samples, implying no mutation in BMPR-1B in our ewes. Regarding the records of twinning in this breed it is concluded that the genetics factor controlling twinning is not related to the mutation, which is reported in Booroola major gene. It may be concluded that litter size in this breed is either not affected by major genes or it is possible that some other major genes control twinning in this breed. Further research is recommended to investigate this**.**

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