INVESTIGATION OF FECUNDITY FecB GENE IN IRAQI AWASSI EWES

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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 (2-3 years) old with an average live body weight 45 kg were obtained from two locations (First , College of Agriculture , second , Agricultural Research Station / Ministry of Agriculture) in Abu-Ghraib , (30 km) south – west of Baghdad, were used in this study from August 2009 to August 2010. Investigation was carried out for multiple fertility gene (FecB) using the (PCR-RFLP) technique. Blood samples (5 ml/ewe) were drawn from the juglar vein using vacutainer tubes with anticoagulant material (ACD). Genomic DNA was extracted from whole blood for each sample using (Wizard genomic DNA purification Kit). The concentration and the purity of extracted DNA were measured with 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) technique. For the purpose of natural point mutation diagnosis in the resulting PCR product is through the digestion of (FecB) gene (190 bp), by using restriction enzymes (*Ava II*). The results of the study showed that: Awassi ewes studied with (++) genotype revealed the absence of fertility genes (FecB). Twining percentage found in some Awassi ewes were not related to this gene and may be are related to other fertility genes or to other factors such as nutrition. No significant differences were observed in the birth weight or body weight at 90 days postpartum.

Key words: FecB gene, Forced PCR-RFLP, Awassi ewes.

التحري عن جين الخصب FecB في نعاج العواسي العراقي

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المستخلص

اجريت هذه الدراسة بهدف التحري عن تعدد المظاهر الوراثية لجين الخصب وتأثيره في بعض مظاهر الاداء التناسلي لدى النعاج العواسي العراقي. استخدمت 82 نعجة عواسي تراوحت اعمارها من 2–3 سنوات وبمعدل وزن جسم حي 45 كغم. تم الحصول عليها من موقعين (الاول كلية الزراعة/ جامعة بغداد والثاني محطة البحوث الزراعية/وزارة الزراعة) الواقعة في منطقة ابو غريب (30 كم) جنوب غربي بغداد للمدة من اب 2009 الى اب 2010. تم التحري عن تعدد المظاهر الوراثية لجين الخصب (FecB) باستخدام تقنية PCR-RFLP. تم سحب عينة دم (5 مل/نعجة) من الوريد الوداجي في انابيب مفرغة من الهواء تحتوي على مادة مانعة للتخثر (ACD). استخلصت المادة الوراثية الدنا (DNA) من الدم الكامل لكل عينة دم باستخدام عدة (Wizard Genomic DNA purification). تركيز ونقاوة المادة الوراثية (DNA) تم قياسها باستخدام جهاز مطياف الاشعة البنفسجية (Spectrophotometry) مع استخدام تركيز هلام (8%) لغرض الترحيل الكهربائي (Electrophorsis). تم عزل ومضاعفة اجزاء محددة من المنطقة المشفرة للمادة الوراثية الدنا (DNA) باستخدام تقنية انزيم بلمرة الدنا المتسلسل (Polymerase chain reaction) وبادئات متخصصة (DNA Markers). ولغرض تشخيص الطفرات الطبيعية النقطية في نواتج التكثير التي تم الحصول عليها من خلال هضم جين (FecB) لازواج القواعد (190bp) على التوالي باستخدام انزيمات التقييد وهي (*Ava 11*). اوضحت نتائج الدراسة ان نعاج العواسي التي تحمل التركيب الوراثي (++) لا تحمل جين الخصب (FecB). نسبة التوائم التي وجدت لدى بعض نعاج العواسي ليس سببها الجينات المدروسة وربما يكون السبب جينات خصب اخرى او عوامل اخرى مثل التغذية. لم تلاحظ فروقات معنوية في الوزن عند الولادة وعند عمر 90 يوماً بعد الولادة.

الكلمات المفتاحية: جين FecB، تقنية PCR-RFLP، نعاج أغنام العواسي.

INTRODUCTION

Iraqi sheep breeds are characterized by low reproductive efficiency and twining rates (23). Litter size and lamb growth are important economic traits in sheep breeding and reproduction (9). Regarding the economic importance of sheep in meat production in Iraq, it becomes essential to make fingerprinting of some genes related to economic traits such as litter size and growth rate in order to determine the polymorphism pattern of these genes in the Iraqi sheep breed. Determination of the genetic diversity of indigenous sheep in Iraq in respect to these important economic genes has not been sufficiently studied. Genetic characterization and determination of genetic differences between sheep breeds will help in the genetic improvement programs. Breeding objectives are needed to develop selection programs for these breeds. Recent studies have reported that the high prolificacy in many prolific sheep breeds around the world is the result of the found FecB gene (7 ; 5). Newly developed DNA tests have encouraged researchers to screen for the presence of these mutations in many local breeds around the world. The Booroola fecundity gene (FecB) is a single autosomal gene, which increases ovulation rate and litter size in sheep, co-dominant for ovulation rate and partially dominant for litter size (15 and 12). The FecB locus is situated in the region of ovine chromosome 6, which is syntenic to human chromosome 4 (13, 3 and 15). 16 and 3 found that the effect of FecB mutation is additive for ovulation rate and each copy increases ovulation rate by about 1.6 and approximately one to two extra lambs in Booroola Merinos. 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 (14 and 22). 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. In recent years, many aspects of the (FecB) gene, including reproductive endocrinology (19), ovarian development (4), litter size, organ development and body mass (20) have been studied. This gene has an additive effect on litter size and ovulation rate (9). Fecundity genes (FecB) 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 (21 and 5). Previous studies have not examined effect of the FecB gene on litter size in Iraqi sheep breeds. Therefore, the objectives of this study were: to investigate the presence of the (FecB) gene by PCR-restriction fragment length polymorphism in Awassi sheep ewes. And evaluate the effects of the (FecB) 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 (30 km south–west of Baghdad), the first location was at the Animal farm/Coll. of Agric. / Univ. 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 Agriculture.

Experimental analysis: A total of eighty two Iraqi Awassi ewes, (2-3) years old with an average live body weight (45 kg) 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 birth and sex of lambs 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 endo parasites.

Blood collection and DNA extraction: Approximately, 5 ml venous blood was collected from each ewe. Blood was placed in tubes containing anti-coagulant solution acid citrate dextrose (ACD) and stored at (20^{o}C). Blood samples were used as a source of DNA for PCR-RELP detection of FecB gene in Awassi sheep ewes. Genomic DNA Extraction

from Frozen Blood (Promega DNA Wizard). The concentration of extracted DNA was measured by using the spectrophotometric method (18). The principle of spectro-photometric method depended on the amount of UV irradiation absorbed by the nitrogen bases composed in the DNA. The method was performed by adding 10 µl of DNA sample to 490 µl of distilled water. Then the optical density was determined at 260 nm in a UV spectrophotometer using distilled water as a blank. The DNA concentration was calculated according to the equation:

DNA conc. µg / ml = O.D 260 nm x Dilution Factor x 50

To determine the degree of contamination of the DNA with protein, an additional measurement was made at 280 nm and the ratio A260 / A280 was calculated. Pure DNA would give an A260/A280 ratio of 1.8 or higher. Values for A260/A280 of less than 1.8 indicated contamination of the DNA with protein for an A260/A280 values of 1.5, the percentage of protein in the DNA preparation is about 50% . For good PCR results, DNA was required with an A260/A280 ratio of 1.6 or greater. The purity and concentration of the DNA was of a crucial importance for optimal results. This ratio was used to detect nucleic acid contamination in protein preparations. DNA quality can be also assessed by simply analyzing the DNA by agarose gel electrophoresis (11).

DNA purity ratio = OD260/OD280

Detection of the FecB gene in Awassi sheep by PCR-RFLP

PCR Primer

Polymerase chain reaction (PCR) was carried out using a modification of the forced restriction fragment length polymorphism (RFLP) method described by (5).The primer reverse CAAGATGTTTTCATGCCTCATCA ACAGGTC) has been engineered to introduce a point mutation resulting in PCR products with FecB mutation containing an *Avall* restriction site (G/GACC) , whereas products from noncarriers lacki this site. (The (190bp) product was digested by *Avall*. Products containing the FecB mutation were digested to yield a (160bp) fragment, whereas non-carriers products remained uncut at (190bp). Primer ready to use and the sequence of this primer are listed in (table 1).

Table 1. Primer and PCR conditions of the candidate FecB gene and enzyme.

PCR amplification of FecB gene

Primer for FecB	Forward	Reverse
Oligonucleotide Sequence 5`-3`	CCAGAGG ACAATAG CAAAGCA AA	CAAGATGT TTTCATGCC TCATCAACA GGTC
Annealing temperature ^{o}C	60	
Restriction enzyme	Avall	
Reference	5	

The preparation of PCR reaction mixture for the printer pair that amplifies a certain region within the FecB was carried out using the following reagents that were mixed in a sterile 1.5 nl eppendorf tube as given in (table 2). Master Mix includes (Taq DNA polymerase , dNTPs $MgCl_2$ and reaction buffers at optimal concentrations for efficient amplification of DNA templates by PCR), was performed in order to achieve homogeneity of reagents and reduce the risk of contamination. Negative control reaction was run in parallel. 12.5 µl of master mix was aliquoted into 0.2 ml eppendorff tubes 2 µl of DNA template was added and mixed gently. All the processes were performed on ice in aseptic conditions using laminar air flow hood.

Table 2. Reagents and their addition order used in FecB PCR amplification

Addition order	Component	Volume µl	Final concentration	Company
1	Sterile D.W	6.5		Promega
2	Promega green mix	12.5	1x	Promega
3	Primer : forward	1.5	10 pmol	Alpha DNA
4	Primer : reverse	1.5	10 pmol	Alpha DNA
5	MgCl2	1.0 / 23	32 mM	Promega
6	DNA (50ng/µl)	2.0 + 23	----	-----
Total	----------	25	----	-----

The amplification program were as follows: Initial denaturation at 94^{o}C for 5 minutes, followed by 30 cycles of Denaturation at 94^{o}C for 30 seconds, Annealing at 60^{o}C for 30 seconds and Extension at 72^{o}C for 30 seconds, with final extension at 72^{o}C for 5 minutes, then hold at 4^{o}C. The PCR reactions were tested by horizontal 2.5% agarose gel electrophoresis.

PCR-RFLP FecB

Restriction Enzyme *AvaII* (Promega, Madison, WI, USA) Digestt 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 , deionized water	11.8 µl
RE 10X Buffer	2 µl
Acetylated BSA, 10 µg / µl	0.2 µl
DNA, Mix by pipetting, then add:	5 µl
Restriction Enzyme (Avall)	1 µl
Final volume	20 µl

Mix gently by pipetting, close the tube and centrifuge for a few seconds in a micro-centrifuge. The reactions then were incubated for at least 4 hours or overnight at the specified temperature for the enzyme (usually 37^{o}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 (Promega Madison, U.S.A). One pocket per row was used for the length standard (50bp DNA ladder) Bench top, volume 4 µl 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 FecB gene produced a 190 base pair (bp) band. The FecB gene homozygous carriers could be identified as having a 160 bp band (BB), the non-carries as having a 190 bp band (++), whereas the heterozygotes as having both the 160 bp and 190 bp bands (B+).

RESULTS AND DISCUSSION

DNA Isolation and Quantitation

The DNA was extracted from eighty two blood samples efficiently by using (Wizard Genomic DNA purification kit). Purity and concentration measured by using the standard method (18). DNA was successfully extracted. The range of DNA concentration extracted from whole blood was (2.7-3.3 µg/µl) and the purity range was (1.2-1.6). The PCR technique does not require large quantities and high purity of DNA (17). Therefore, the resulted DNA concentration and purity have been suitable for amplification using PCR technique.

Detection of the FecB mutation gene

According to the results found in the present study , it was revealed that the all samples had (++) genotypes. The resulted PCR products were digested with *AvaII* for FecB luci and genotypes of each individual were detected by electrophoresis. Restricted digestion of PCR products with FecB with *AvaII* restriction enzyme have not showed a mutation, where the A nucleotide has changed to G nucleotide at this locus. Result with digestion was only one fragment 190bp (Figure 1).

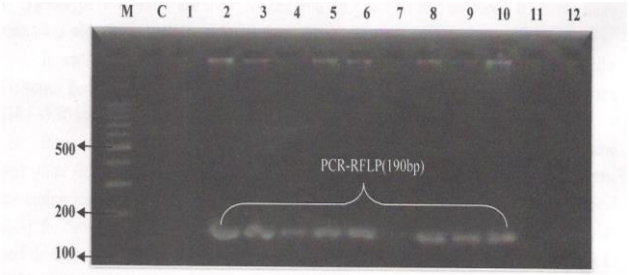

Figure 13: PCR amplification of FecB gene (190 bp,Lanes 2-6) in Awassi sheep breed and digesation product of samples with *AvaII* restriction enzyme (lanes 8-10) with genotype ++ . Lane C, negative control and Lane M, molecular size marker (100 bp DNA ladder). Bands were fractionated by electrophoresis on a 2.5% agarose gel (95min, 5V/cm, 1XTris-borate buffer) and visualized under U.V. light after staining with ethidium bromide.

The results showed that the frequency of polymorphism distributions of FecB mutation gene was imbalanced in breed (Table 3). All genotyped sheep had the wild type allele (++).

Table 3. The frequency distributions of FecB gene in Awassi sheep breed

Gene	FecB		
Number of ewes	82		
Allelic frequency	+	B	
	1	0	
Genotypic frequency	++	B+	BB
	1	0	0

RELP is a rapid, simple and exact technique for single nucleotide polymorphism (SNP) genotyping. After a forced restriction site was introduced into one of the primers. The PCR product contained a certain restriction enzyme site. This forced PCR-RFLP approach has been used previously to genotype prolific sheep (21 and 5) and swine (10) to decide whether they had the same mutation as FecB in Booroola Merino sheep. In our study , PCR-RFLP approach was used to detect the genotype based on the method described by (21) and (5) . RFLP has a good repeatability and stability, but its results were affected by several factors, such as enzymes from different

companies, time of digestion, volume of electrophoresis and concentration of gel. We compared several of these factors by adding various concentrations of ingredients for selecting the optimal reaction conditions to maintain repeatability and veracity. Detections with illegible results were repeated until the genotyping was clear. A total of eighty two blood samples individuals from Awassi sheep were genotyped with the PCR-RELP approach (Figure 1). The results showed that the frequency of polymorphism distributions of FecB gene was imbalanced in eighty two blood samples. All genotyped ewes in this study had the wild type allele (++), these results are in agreement with reports in 7 of 9 sheep breeds in China studied were found to be wild type (190 bp++) in respect to restriction pattern of FecB gene (9). On the other hand ,(9) pointed out that Chinese Merino prolific meat strain had the three different Booroola genotypes (BB, B+ and ++), while Hu-sheep in China were all homozygous for FecB (BB), in addition , they found that Hu-sheep crossbreed progeny had B+ genotype, which exhibited a simple Mendelian pattern of segregation when they were backcrossed, and reported positive relationship between mutation of FecB gene and litter size in Chinese Merino prolific meat strain, where the litter sizes of ewes with BB Genotype averaged (2.84 $\pm$ 0.74), which was significantly greater than those (1.23 $\pm$ 0.68) of ewes having (++) genotype ($P < 0.01$), and the Hu-ewes with BB genotype also produced 0.5 lambs more than ewes with (B+) genotype, although the difference was not statistically significant. In addition, (9) found at 90 days after birth, the body weights of the Hu-ewes genotypes (BB, B+) lambs were higher than those Hu-ewes genotype (++) lambs (18.6 $\pm$ 3.70 kg), (18.0 $\pm$ 3.71 kg) versus (15.6 $\pm$ 2.22 kg), respectively, ($P < 0.05$). (15) Explained the positive relationship between mutation of FecB gene and litter size when the effect of FecB gene was additive for litter size. Ovulation rate, increasing corpora lutea by about 1.65 per / copy and litter size by (0.9) for one copy and (0.4) for two copies. On the other hand, it has negative effects on fetal body weight, body size and development during pregnancy, body weights were lighter at most gestational ages in (BB) , (B+) than those (++) fetuses, and the BB/B+ grew slowly and body lengths were shorter than (++) fetuses (19). The results in this study showed that the presence of non-carrier (FecB gene) 190bp band pattern (wild type) in all the animals studied could be explained on the basis of low litter size in Awassi breed, since the presence of (++) wild type is significantly correlated with low litter size as reported by (9 and 1). This mutant gene may be transmitted to the other breeds via crossbreeding in a number of countries. Awassi breed, for example, is characterize at low lambing percentages, lambing which the Booroola gene has been transmitted and fixed since, 1986, the fertility rate of this breed increased from (1.2-2) lambs per lambing without significant decrease of milk production (2 and 8). In this study results of PCR-RELP method showed the same band pattern in all (82) blood samples, implying no mutation in FecB locus in Awassi ewes due to small size of the sample studied in this research , there is a probability that the mutant allele was not available in the sample. Therefore, there is a need to undertake a further research on a relatively larger size sample for the population. A number of other mutant genes affecting lambing. Regarding the records of twinning percentage in Awassi breed it is concluded that the genetics factor controlling twinning percentage is not related to the mutation, which is reported in Booroola major gene, may be litter size in Awassi breed is either not affected by major genes or it is possible that some other major genes control twinning percentage in this breed.

Conclusion

It can concluded that Digestion of FecB gene 190 base pair with *AvaII* restriction enzyme resulted in non-carrier 190 bp band (wild type) in all the animals belonging to the breed studied revealing absence of this restriction site in this sheep breed. The presence of non-carrier 190 bp band pattern (wild type) in all the animals belonging to the breed studied could be explained on the basis of low litter size in this breed, since the presence of ++ wild type is significantly correlated with low litter size. Further studies should be made to determine required crossbreeding ratio with foreign breed carry the favorite genotypes of

this gene which will lead to increase of expression of this gene in the local Iraqian breed without effect on the acclimatization traits of this breed to the environmental conditions in Iraq.

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