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Investigation of the Booroola Gene in Egyptian Ewes with Different Reproductive Status Using PCR- RFLP

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OVULATION CAN BE regulated by a group of genes, termed as fecundity (Fec) genes. The aim of this study is to identify genetic polymorphism in the Booroola (FecB) gene in Barki, Rahmani, and Ossimi sheep breeds with different physiological status diagnosed by ultrasound. Accordingly, animals were early classified into three groups: carrying single fetus, twin fetuses and non-pregnant by ultrasonographic examination. Demonstration of the fetal number was available as early as Day 35-40 post-mating transrectally, and trans-abdominally. The fetal viability was checked through heart examination with M-Mode. Genomic DNA was extracted from blood samples of the total number of sheep and two primers were used to amplify 190 and 140 bp fragments of *FecB* gene. The amplified fragments were digested using *AvaII* restriction enzyme. All sheep groups were non carriers for the *FecB* mutation and gave a 190 bp band (++) and 140 bp band (Fec++) for primer 1 and 2, respectively. In conclusion, no genetic polymorphism was detected in the three Egyptian sheep breeds in relation to pregnancy with single or twin fetuses. The study could be continued to search for other major genes.

Keywords: Sheep, Booroola (FecB) gene, Genotyping, Ultrasound.

Ultrasound is important in animal reproduction examination since 1980, offering both a mean of diagnosis and a useful therapeutic tool. Sonography or B-scan-real time echography permitted a reliable early pregnancy diagnosis in sheep and goats (Fowler and Wilkins, 1980).Generally, the findings on the non-pregnant uterus as well as the uterus and conceptus during pregnancy are similar in sheep and goats (Tainturier *et al.,* 1983). Pregnancy diagnosis in sheep and goats can be made early at 25 days post breeding using real-time ultrasonography (Buckrell, 1988). In goats, Hesselink and Taverne (1994) found that trans-abdominal scanning between days 40 and 70 after mating, yields information about fetal livability and single or multiple pregnancy.

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In sheep and goats, twining and triplicate fetuses are common. Several studies had been conducted on genes controlling ovulation rate and prolificacy in prolific sheep breeds. Mainly, the three fecundity genes of bone morphogenetic protein receptor type 1B (BMPR1B) or activin-like kinase 6(ALK6), also named Booroola or FecB (Souza *et al.,* 2001); bone morphogenetic protein 15 (BMP15) known as FecX on chromosome X (Hanrahan *et al.,* 2004 and Barzegari *et al.,* 2010) and growth differentiation factor 9 (GDF9), known as FecG on chromosome 5 (Hanrahan *et al.,*2004) had been identified in sheep. Mutations found on these genes had been associated with different phenotypic as the FecB mutated allele was correlated with an additive effect on the rate of ovulation and increase in litter size (Souza *et al.,* 2001, Davis, 2004 and Kumar *et al.,* 2006).

In sheep, the BMPR 1B gene had been mapped to chromosome 6 with coding sequence of 10 exons (Mulsant *et al.,* 2001). G/A transition mutation at nucleotide 746 of BMPR 1B cDNA, had been associated with the "hyperprolific phenotype" in Booroola sheep (Souza *et al.,* 2001 and Wilson *et al.,* 2001).

Knowledge about these mutations has encouraged researchers to screen other prolific sheep breeds to know whether these mutations is responsible for their high prolificacy. A wide study of twenty-one of the world's prolific sheep breeds revealed presence of FecB mutation in only two breeds (Hu and Han) from China (Davis *et al.,* 2006). But reports from Egyptian, Tunisia, Iraqi sheep breeds did not record any FecB mutation (Al-Barzinji & Othman, 2013 and Elkorshy *et al.,* 2013). So, the present work aimed to detect the mutation in FecB gene in Barki, Rahmani, and Ossimi Egyptian sheep breeds in different physiological status which were detected by ultrasound.

Material and Methods

Animals

A total number of 68 Barki ewes kept in a private farm at Gamgara village, Benha, and 47 sheep local breeds (Ossimi and Rahmani) raised in South Sinai-Egypt, were used in this study. Ewes averaged 41.50 ± 3.75 kg body weight and 3-5 years age. All animals were apparently healthy and free from parasites and were kept under natural photoperiod and ambient temperature. Ewes were fed on a ration contained 60% concentrate feed mixture (composed of 30% wheat bran, 15 % cotton seed meal, 35 % yellow corn, 15 % sunflower meal, 3 % molasses, 1.5 % limestone and 0.5 % salt) plus 40 % clover and rice straw twice a day according to NRC (1989).

Ultrasonographic examination of pregnancy

Animals were investigated with ultrasonography prior to blood sampling using a B-mode ultrasound machines (Eickemeyer Magic 2200, Germany, equipped with a 4-6 MHz ultrasound linear probe; SonoScape A6V, China, equipped with 12 Mhz Linear transducer and 6.5 MHz micro-convex transducer). For trans-abdominal approach, the ventral of the abdomen was clipped and shaved carefully using clippers. The well lubricated ultrasound probe was

positioned in the groin area close to the anterior margin of the udder and progressed cranially while the animals were standing to allow complete examination of the uterus and its contents (Kandiel *et al.,* 2016). The numbers and viability of fetuses were detected by ultrasonography. Accordingly, ewes were categorized into three groups: carrying single, twin fetuses or non-pregnant.

Blood collection and DNA isolation

Blood samples were collected, separately, using the anticoagulant EDTA in vacationer tubes and transported to the laboratory under cooled conditions for optimization the PCR procedure. Genomic DNA was extracted from blood samples with the Reliaprep^{TM} DNA blood kit (Promega, USA) according to the manufacturer's instructions. The DNA samples had the ratios 260/280 OD in the range of 1.8 to 2, indicating high purity. DNA was also examined on 1% agarose gel and visualizing the band under gel documentation system.

PCR reaction

Amplification reactions of 50 µL containing 5µl buffer 10x, 1µ 2.5 mM (dNTPs mixture), 3µl 25 mM ($MgCL_2$), 0.25µl primer, 0.3µl Taq polymerase (5U/µl), 35.2 µl nuclease free water, 5µl DNA sample were done. The reaction was cycled for 1 min at 94°C, 30 sec at an optimized annealing temperature that was determined for each primer (Table 1) and 2 min at 72°C for 30 cycles. After the reaction was completed, PCR products were subjected to electrophoresis in2.5% agarose gel, 1X TBE buffer with ethidium bromide, at 60 V for approximately 2.5 hrs. Visualization of the bands was done under ultraviolet Trans-illumination and the pictures were taken in Gel-Doc equipment (Bio-Rad).

TABLE 1. The identification of the primer and restriction enzymes of FecB gene.

Primer	Primer sequence (5' – 3')	Annealing temperature	Size (bp)	Restriction enzyme	Reference
1	CCAGAGGACAATAGCAAAGC AAA CAAGATGTTTTCATGCCTCAT CAACAGGTC	60°C 30 sec	190 bp	*AvaII*	(Davis *et al.,* 2002)
2	GTCGCTATGGGGAAGTTTGG ATG CAAGATGTTTTCATGCCTCAT CAACACGGTC	60°C 30 sec	140 Bp	*AvaII*	(Wilson *et al.,* 2001)

Forced restriction fragment length polymorphism

For genotyping, the PCR products of the two primers were digested with *AvaII* (Fermentas). Gene fragments was subjected to digestion by restriction enzymes in 20 µL volume contained 10 µL reaction solution, 2µL enzyme buffers, 0.2 µL enzymes, and 7.8 µL water and placed in the thermocycler for 1 h at 37 °C. After digestion samples were quantified to visualize the amplified fragments by gel electrophoresis as mentioned in PCR.

Primer 1: The PCR of the *FecB* gene produced a 190 base pair (bp) band. After digestion with *Ava*II, the *FecB* gene homozygous carriers had a 160 bp band (BB), the noncarrier had a 190 bp band (++), whereas heterozygotes animals had both 160 and 190 bp bands (B+).

Primer 2: The PCR product of the FecB gene produced a 140 bp band. After digestion with *AvaII*, the FecB gene homozygous carriers should produce a 110 bp band (FecBB), the non-carrier should produce a 140 bp band (Fec++), whereas heterozygote animals should produce both 110 and 140 bp bands (FecB+).

Results

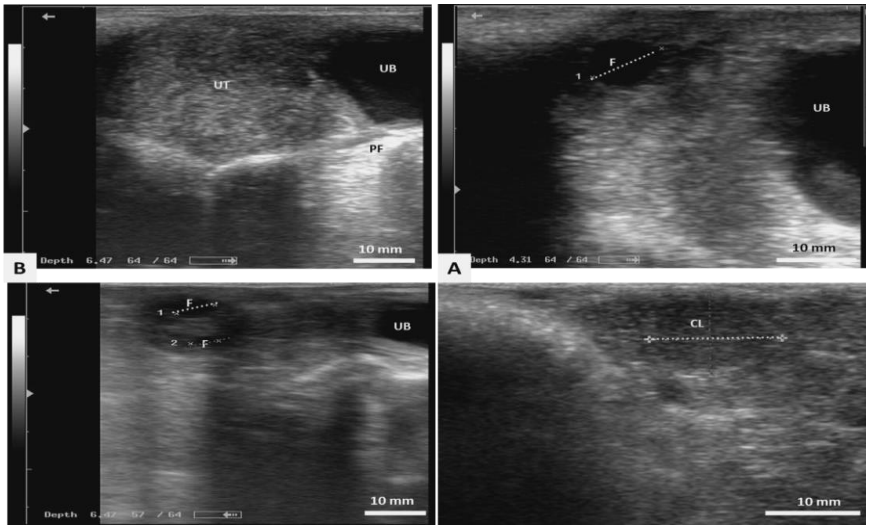
Representative ultrasonographic images of sheep ovaries and non-pregnant uteri were presented in ultrasonogram 1. Non-pregnant uterus of ewes appeared as a homogenous, coarsely granular hypoechoic tissue lied on the hyperechoic pelvic floor at the apex of the bladder. Ovarian with its structures either ovarian follicles (circumscribed anechoic structure) or corpus lutem (irregular hypoechoic structure) have been observed on both sides of the uterus with rotation of the ultrasound probe at angle 45-90 degree.

The position of the pregnant uterus was variable according to the stage of pregnancy therefore was demonstrated at first rectally up to Day 35-40 after breeding and trans-abdominally thereafter. Representative ultrasound images of sheep uteri contained single or twin fetuses are presented in ultrasonogram 2.

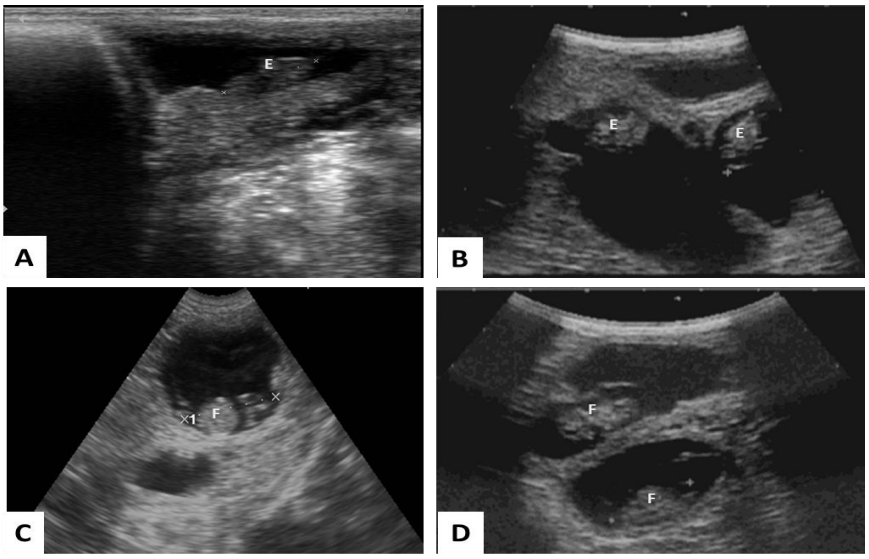
Symbolic ultrasound images of fetal viability and stage of pregnancy determination are demonstrated in ultrasonogram 3. Determination of fetal viability has been done through heart examination with M-Mode as it appeared as a hypoechoic moving/contacting structure at the apex of the thorax. At mid-stage of pregnancy, the echogenic fetus was surrounded with a large amount of the anechoic fetal fluid. Placentomes with a characteristic C-shape (longitudinal section) or sickle shape (cross section) hypoechoic appearance were dominant in most of the ultrasonographic pictures during scanning of pregnancy. Fetal head with its characteristic hypoechoic fetal brain and anechoic ocular cavity was visible in different alignments inside the pregnant uterus.

The PCR-RFLP technique was used to study the polymorphism of BMP1B receptor (FecB) gene in a total number of 115 sheep including 68 Barki, 47 Ossimi and Rahmani. The PCR amplified a fragment of 190-bp in size and 140-bp product of two different primers for the same gene were obtained in all sheep.

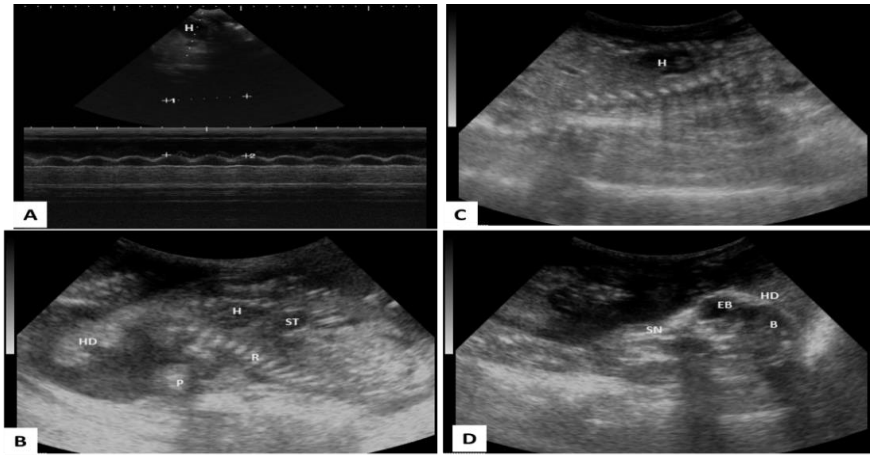
Both PCR amplified fragments of 190-bp and 140-bp were digested with Eco471 (*Ava II*) endonuclease. Depending on the presence or absence of the restriction site, all samples in this study are genotyped as AA for PCR amplified fragments at 190-bp (Fig.1) indicating absence of Ava II restriction site in all breeds. While PCR amplified fragments at 140-bp were digested with Eco471 endonuclease at which there is one pattern obtained in all studied sheep (Fig.2) and genotyped BB as the wild type fragment. The results showed that all animal groups for this locus were monomorphic.



Ultrasonogram 1. Representative ultrasonographic images of the uterus and ovaries innon-pregnant ewes. UT: uterus. UB: Urinary bladder, F: Follicle and CL: corpus luteum.



Ultrasonogram 2. Representative ultrasound images of sheep uterus contained single or twine fetuses. E: embryo. F: Fetus.



Ultrasonogram 3. Representative ultrasound images of fetal viability and stage of pregnancy determination in ewes. A: B-mode (upper panel) and M-Mode (Lower panel) of fetal heart rate examination. H: Heart. HD: Head. ST: Stomach. SN: Snout. R: Ribs. P: Placentome. B: Brain.

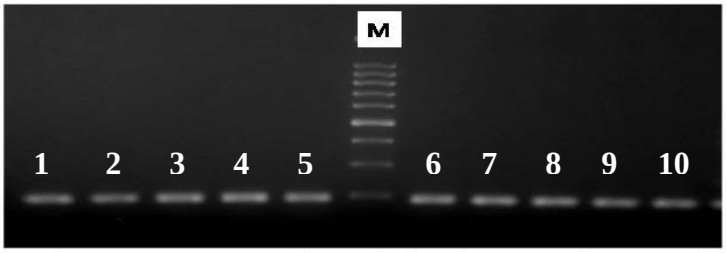


Fig. 1. DNA electrophoretic pattern was obtained after digestion of PCR amplified sheep of FecB gene product with *Avall*. M 100 bp ladder marker, lanes from 1-10 showed undigested band (190 bp).

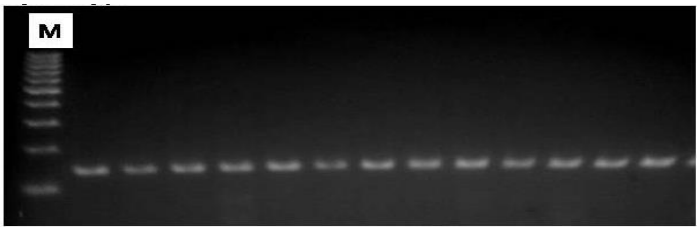


Fig. 2. DNA electrophoretic pattern was obtained after digestion of PCR amplified sheep of FecB gene product with Avall. M 100 bp ladder marker, lanes from 1-13 showed undigested band (140 bp).

Discussion

The practice of ultrasound techniques for diagnostic purposes in veterinary medicine enables the visualization of ovarian and uterine structures to delineate the normal and pathological conditions (Taverne and Willemse, 1989). The parameters adopted in the current study to monitor the fetal viability and numbers as well as progression of normal pregnancy were in accordance with that reported in Awassi × Marino (Karen, 2003) and Barki (Kandiel *et al.,* 2016) breeds of sheep. Determination of the fetal number, single and multiple, using ultrasonography pregnancies in sheep were accurately detected on Day 25 transrectally (Schrick and Inskeep, 1993) and at Days 46 to 93 trans-abdominally (White *et al.,* 1984). Fetal viability determination based on examination of fetal heart rate was validated within the ovine embryonic vesicle using ultrasound waves of 7.5 MHz on Day18 or 19 (Schrick and Inskeep, 1993) and 5 MHz from Day 21-23 (Garcia *et al.,* 1993). Likewise, a remarkable correlation was found between fetal heart rate and gestation stage (Karen *et al.,* 2001). These findings indicted the role of ultrasonography in offering an assessment of pregnancy condition and fetal viability early after breeding in order to ascertain the animals that fail to conceive, improving reproductive efficacy, early recognition of those carrying twin fetuses. This allows the implementation of distinct management policies to avoid the undesirable impacts of twinning on general health of the dam and also at lambing (Medan and Abd El-Aty, 2010).

The amplified fragment of the FecB gene for primer 1 resulted a 190 bp band after digestion with *AvaII* restriction enzyme. Similarly, El-Hanafy and El-Saadani (2009) reported that digestion of 190 base pair FecB gene with Ava II produced 190 bp band in non-carrier(++)wild type for Egyptian sheep breeds of Rahmani, Awassi, Barki, Ossimi and Awassi x Barki. Also, Elkorshy *et al.* (2013) did not show the FecB mutation for the BMPR-1B gene in Egyptian (Barki, Ossimi, Rahmani) and Saudi sheep (Najdi and Harri). In both Egyptian studies, the 190 base pair of FecB gene was amplified to introduce mutation in FecB carrier sheep containing an *AvaII* site (G|GACC), whereas non carriers lacked this restriction site. In contrast, the FecB gene polymorphism in Irananian Zel breed sheep was detected by Asadpour et al. (2002) who reported genotype frequencies of BB (0 %), B+ (1.47 %) and ++ (98.53 %). Moreover, the genotype frequencies of BB, B+ and ++ were 51%, 30% and 19%, respectively in the Chinese Merino prolific sheep (Guan *et al.,* 2007) and they added that FecB gene had a positive effect on litter size and early postnatal body growth.

In the current work, digestion of the *FecB* gene 140 bp in primer 2 with *AvaII* restriction enzyme produced 140 bp band in noncarrier (++) wild type in Barki, Rahmani and Ossimi Egyptian breeds. These results are in agreement with reports in Egyptian sheep (Abulyazid *et al.,* 2011), and five breeds in Iraqi sheep as Hamdani, Karadi, Awassi, Naeimi and Arabi (Al-Barzinji and Othman, 2013). Moreover, data from North African revealed absence of FecB mutation in sheep

breeds reared in Tunisia as Barbarine, Queue Fine deL'Ouest, Noire de Thibar, Sicilo-Sarde and D'man (Vacca *et al.,* 2010).

However, our data contrast with other studies in Booroola Merino (Wilson *et al.,* 2001), Hu and Han sheep Chinese Merino prolific meat strain (Davis *et al.,* 2006) and Kendrapada Indian sheep (Kumar *et al.,* 2008). It has been known that Hu and Chinese Merino prolific sheep were highly prolific, and Chinese Merino and Romney hills breeds were more prolific compared with other breeds. The phenomenon probably due to the gene being fixed in the Hu population, as in some Garole sheep in India (Davis *et al.,* 2002). Moreover, the FecB mutation was reported in the Nilagiri sheep, India with the 0.14 frequency (Sudhakar *et al.,* 2013). The Nilagiri sheep had been recorded as the third prolific in India after the Garole and Kendrapada.

Absence of mutation in Egyptian sheep breeds could be explained the low litter size for these breeds, as the presence of ++ wild type was related to low litter size (Guan *et al.,* 2007). In this context, Galal *et al.* (1996) recorded that sheep breeds in Egypt are medium size, breed all the year, low growth rate and had small litter size ranging from 1.03 to 1.40.

The absence of prolificacy genotypes in our sheep breeds implies that these mutations affecting prolificacy may be added to our breeds by genetic introgression which allows the introduction of a desirable genotype in Egyptian breed (Hua and Yang, 2009). An example of FecB mutation introgression, the crossbreeding of Garole×Malpura allowed the introgression of the FecB genotype in Garole sheep into non-prolific Malpura, to improve the litter size of their crossbreds (Kumar *et al.,* 2006).

Conclusions

None of Barki, Rahmani and Ossimi sheepbreeds carried *Fec* B mutation and more screening is required to discover new mutations with increasing animal numbers.

Declaration of interest: The authors declare that there is no conflict of interest.

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دراسة جين البورولا في الأغنام المصرية خلال المراحل التناسلية المختلفة باستخدام PCR- RFLP

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يتم تنظيم التبويض في إناث الحيوانات بواسطة مجموعة من الجينات تعرف بجينات الخصوبة وتهدف الدراسة الحالية إلى تحديد الطرز الوراثية لجين البورولا (Fec B) في سلالات الأغنام البرقي والرحماني والأوسيمي فى المراحل التناسلية المختلفة والتي يتم تشخيصها بواسطة الأشعة الفوق صوتية . تم تقسيم النعاج إلى عشار تحمل جنين واحد وأخرى تحمل توأمين ومجموعة ثالثة غير عشار. وقد أمكن تحديد الأجنة عند عمر ٣٥- ٤٠ يوم من التلقيح والكشف عن حيوية الأجنة بفحص القلب بواسطة السونار. وقد استخلص الحمض النووي (DNA) من عينات الدم لكل النعاج و عمل تقنية تفاعلات البلمرة المتسلسل (PCR) باستخدام بادئين نتج عنهما قطعتين طولهما ١٤٠ و١٩٠ نيوكليتيدة. وقد وجد أن جميع الأغنام لا تحمل طفرة (Fec B) وأن النتائج توضح عدم وجود طفرات ترتبط بزيادة التوائم في سلالات الأغنام المصرية الثلاثة التي تم دراستها.

الكلمات الدالة: الأغنام - البورولا جين - الطرز الجينية - السونار.