Animal Reproduction Science 208 (2019) 106113

Contents lists available at ScienceDirect

Animal Reproduction Science

journal homepage: www.elsevier.com/locate/anireprosci

Novel SNPs in the *SPAG11* gene and association with testicular biometric variables in Boer goats and application of the levelled-container technique

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ARTICLE INFO

Keywords:
Levelled-container
Testicular volume
SNP
SPAG11
Goat

ABSTRACT

Testicular volume (TV) is one of the most important traits used in evaluation of the reproductive capacity of male animals. The levelled-container used in the present study was found to be reliable instrument to measure TV, based on a water displacement method. Sperm-associated antigen 11 (*SPAG11*) is an important gene that affects male reproductive performance. An objective of the present study, therefore, was to determine if single nucleotide polymorphisms (SNPs) in a fragment of the *SPAG11* gene could be used to determine associations with values of testicular biometric variables in Boer goats. Primers were designed to amplify the full length of the first two exons of *SPAG11*. The targeted fragment was generated using a molecular cloning technique. As the result, four SNPs, [g.1256A > G(*ss19199134542*), g.1270C > T(*ss19199134541*), g.1325A > G(*ss19199134540*) and g.1327 G > A (*ss19199134543*)], were detected using a single-base extension (SBE) method. Two of these SNPs were synonymous (*ss19199134540* and *ss19199134542*). The other two SNPs were nonsynonymous, thus, there were changes in amino acid in the resulting protein: threonine to isoleucine (for *ss19199134541*) and arginine to glutamine (for *ss19199134543*). The SNP *ss19199134543* was the only locus detected that was associated with TV ($P = 0.002$). None of the testes dimensions nor TW were associated with detected SPAG11 gene SNPs. Most likely, the *ss19199134543* locus affects tissue structures adjacent to the testes, causing the change in TV. In conclusion, among the studied testicular biometric variables, TV had the greatest potential for preselecting of bucks with desirable semen quality. The use of the levelled-container as a TV measurement approach was an accurate and reliable method.

1. Introduction

Testicular volume (TV), whether in isolated or non-isolated testes, is an important trait in animal reproduction studies (Gouletsou et al., 2008; Pant et al., 2003). The TV is used as a common trait for evaluating fertility (Kastelic and Thundathil, 2008). As a component of animal husbandry, therefore, only the non-isolated TV is considered in the present study.

The TV can be measured using two main procedures, namely, by water displacement based on the Archimedes principle and by testicular dimension methods (Abdullahi et al., 2012). From an animal welfare perspective, the water displacement procedure is more

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https://doi.org/10.1016/j.anireprosci.2019.106113
Received 20 January 2019; Received in revised form 19 April 2019; Accepted 26 June 2019

convenient and, therefore, has been applied to assess breeding soundness evaluations (BSE) (Raji et al., 2016), body condition scores (BCS) (Yakubu and Musa-Azara, 2013), semen quality evaluation (Condorelli et al., 2013), and Gonad Somatic Index (GSI) (Eljarah et al., 2017). One particular instrument used to measure TV is an overflow vessel along with a graduated cylinder. Because this equipment is not designed for measuring TV on farms, difficulties arise when handling both pieces of equipment during measuring and ascertaining the volume while maintaining a level positioning of the vessel (Gupta, 2002; Valenstein et al., 2006). In addition, with this method the presence of at least two trained personnel is always required to operate the equipment. The use of the overflow vessel is also not a rapid and efficient method to conduct. When immersing the scrotum inside the overflow vessel, there is considerable time required to allow for the entire amount of water to drain into the graduated cylinder. Even with these limitations, the TV is an important reproductive variable to be ascertained in farm animals (Strina et al., 2016), hence, a more user-friendly method needs to be developed. A modified beaker which is termed “levelled-container” was designed to determine TV. An assessment of the reliability and efficiency of use of this instrument for determination of TV, however, is an important aspect if broad use of this approach is going to be implemented in goat production enterprises (Kanakasabapathy et al., 2017).

The TV determination using the water displacement method is not an actual TV assessment (Lin et al., 2009) because it includes the volume of the anatomical components that are attached to testes such as the spermatic cord, vas deferens, epididymis, and the parietal layer of the tunica vaginalis. The TV, as a male reproductive trait, is an indicator for seminogram variables. It, therefore, has become a very important criterion for evaluation of the reproductive capacity of male animals (Pant et al., 2003).Furthermore, Testicular weight (TW) is also known as an indirect indicator of semen production capacity (Marai et al., 2008). During the past few decades, semen production traits have become variables on which there is focus by animal breeders. To obtain more accurate predictions in genetic selection programs, especially with regard to characteristics which are difficult to measure such as semen quality traits with low to moderate heritability (between 0.19–0.39) (Boligon et al., 2010), personnel of breeding/genetic enterprises are taking advantage of several techniques; such techniques include the estimated breeding value (EBV) (Henderson, 1984), which is based on progeny testing or, more recently, the genomic estimated breeding value (GEBV) which is based on a combination of a marker-assisted selection (MAS) and phenotypic information (Hayes et al., 2009; Hu and Yang, 2014; Khare and Khare, 2017).

Many researchers have investigated the association between TV (Fortes et al., 2012), TW (Cui et al., 2018; Zhao et al., 2016), and single nucleotide polymorphisms (SNPs) within candidate genes to predict the quality and characteristics of semen. A candidate gene is a known gene or variation of a gene which has a significant effect on the particular trait(s). Sperm-associated antigen gene 11 (*SPAG11*) is a candidate gene which codes for sperm-associated antigen proteins, and it is also termed epididymal protein 2 (EP2). Notably, this protein has an important function in the male reproductive tissues (especially in the epididymis) by affecting β-defensin peptide synthesis (Ribeiro et al., 2012). Furthermore, there is an association between the *SPAG11* gene SNPs and quality of fresh and post-thaw semen in Boer goats (M.F. Harighi, unpublished doctoral dissertation, April 2019).

The initial approach for determining if there is a particular molecular marker related to a specific trait is the study of the association between that candidate gene and the expression of a desired phenotype which occurs by conducting what is termed an association study (Vignal et al., 2002). The present research, therefore, focused on conducting an association study between some testicular traits and the *SPAG11* gene with the following objectives:

1) To assess the reliability and validity of the levelled-container, compared with a conventional overflow vessel, for goat TV measurement through a comparison study.
2) To investigate the association between the *SPAG11* gene polymorphisms and several testicular biometric variables (e.g., TV, TW, testis dimensions and thickness of the scrotal skin).

2. Materials and methods

The animal experiment was performed using the protocols approved by the Institutional Animal Care and Use Committee at University Putra Malaysia.

2.1. Experimental animals and blood samples

Mature Boer bucks ($n = 52$) between 18 and 52 months of age were used for this experiment. The bucks were considered to be in desirable body condition and health, and all were maintained in the same enclosure and had access to the same food sources for nutrient intake. Data collection was conducted during one season of the year. The farm was located in the Negeri Sembilan state, Malaysia. To conduct the analysis, the TV of each buck was measured using both the levelled-container and an overflow vessel. From each animal, 5 ml of blood was collected using a Venoject vacuum tube (K2-EDTA, BD Vacutainer®, UK) containing anticoagulant (0.5 mM EDTA, pH = 8), along with a Vacutainer needle and needle holder from the jugular vein. The blood samples were stored at −20 °C. Considering the available pedigree information, care was taken to not include animals in the study with a similar genetic relationship, especially from aspect of sibling relationships (e.g., twin male siblings).

2.2. Use of the levelled-container

2.2.1. Testicular volume experiment using the levelled-container and an overflow vessel

The TV was recorded three times from each buck at 2-week intervals with both a levelled-container and an overflow vessel by a trained operator. To ensure that TV was being consistently measured using the same methods, a preliminary study was conducted

Fig. 1. Prototype of a 1000 ml levelled-container with two bubble level vials.

with three repeated measurements of TV for each of five testicular assessments using both methods. The vessels were filled with 500 ml of water with a temperature of 37 °C. A specific length of the scrotum was marked to ensure that an equal length of scrotum was immersed in both of the vessels. To measure the TV, there was pressure placed on the testes so the testes were positioned at the bottom of the scrotum for measurement purposes. The repeatability of the measurements for each method was assessed using range bars on a graph of plotted data. Because one of the most frequent errors in determining TV is related to the improper (lack of level placement) position of measuring vessels for volume determination (Valenstein et al., 2006), the levelled-container is designed from a 1500 ml plastic beaker with handle that is equipped with two bubble level vials. These were attached in a vertical direction perpendicular to each other to allow for assessment of the stability in roll and pitch of the beaker's vertical axis (Fig. 1). The standard equipment, used for the water displacement technique, was an overflow vessel. With this technique, the overflowing water from the vessel is collected with a graduated cylinder when the scrotum is immersed in the water.

2.2.2. Statistical analyses for levelled-container

Descriptive statistics were obtained using a statistical software (JMP®, Version 11. SAS Institute Inc., Cary, NC, USA) (Inc, 2013). The variables were initially tested for normality using a Shapiro-Wilk test, and outlier data were eliminated. The mean difference in TV values obtained using two different methods was compared using a one-way ANOVA at a significance threshold of $\alpha = 0.05$. Both Passing-Bablok regression and Bland-Altman plot procedures were used to assess the reliability and validity of measuring TV in goats with the levelled-container ($\alpha = 0.05$) (Bland and Giavarina, 2015). A statistical software (MedCalc, version 17.9.2; MedCalc Software, Mariakerke, Belgium) was used for both of these procedures (MEDCALC, 2015). In the Passing-Bablok regression model, the estimated TV values of the levelled-container and the overflow vessel were considered as the y- and x-variables, respectively.

2.3. SPAG11 association study

2.3.1. TV, TW and testes dimensions measurements

The verified leveled-container (through the comparison study) was used to measure TV. The TW was assessed using (Bailey et al., 1996) the following formula: $TW = 0.5533 \times Tle \times Twe^2$, where TW, Tle, and Twe are testicular weight, testis length, and testis width, respectively. Total right and left TW were used for analysis.

The testes dimensions including length, width and thickness (height) were recorded at the time of TV measurements. In addition, the scrotal skin diameter was measured with a dial caliper with an accuracy of ± 0.001 (Seager™, Switzerland). Similar to the TV measurement, the testes dimensions were recorded three times from each buck at 2-week intervals. The measurements were made while the buck was being restrained in a confinement chute.

To measure the length of the testis without the epididymitis, a fixed jaw caliper was placed at the proximal end of the testis, and a movable jaw was placed at its distal end. For the thickness measurement, the fixed jaw of the caliper was placed at anterior side of the testis, and the movable jaw was placed at the thickest point of its posterior side. The width was measured at the widest part of the testis in medial-lateral position (Love et al., 1991). The measurement of the scrotal skin thickness was conducted on the hairy surface between left foreleg and brisket (Fourie et al., 2005). The scrotal circumference was measured while both the testes were laid side-by-side, and pushed tightly into the bottom of the scrotum, using a scrotal circumference tape around it’s the greatest diameter. Care was taken to hold the scrotum in a position where there was no evidence of skin wrinkling.

A flexible tape was used to measure the scrotal length. The measurements were recorded in centimeters to one decimal place and a consistent measuring technique was used for all of the bucks (Williams, 2017).

The following three different formulas were used to compute TV, based on testes dimensions and the diameter of the scrotal skin.

2.3.1.1. *Formula A*.

The Prolate ellipsoid shape (Love, 2014) was used with Formula A to estimate TV where (TV-FA) = $4/3\ \pi \times a \times b \times c$ or with the simplified formula of $L \times W \times H \times 0.52$.

2.3.1.2. *Formula B*.

The Empirical shape (Lambert formula) (Mbaeri et al., 2013) was used to estimate TV with Formula B where TV (TV-FB) = $L \times W \times H \times 0.71$.

2.3.1.3. *Formula C*.

The Prolate spheroid shape (Hansen-Bessel formula) (Mbaeri et al., 2013) was used to estimate TV with Formula C where TV (TV-FC) = $L \times W2 \times 0.52$ where a = thickness/2, b = width/2, c = length/2, $\pi = 3.14159$ and $c < b < a$; L = length of testis W = Width of testis H = thickness (Height) of testis.

Testes dimensions were adjusted by the corresponding diameter of scrotal skin. Total volume of right and left testes was considered as the calculated TV.

2.3.2. *DNA extraction*

The genomic DNA was extracted from whole blood using a DNA extraction kit (DNeasy® Blood and Tissue kit, Qiagen, Hilden, Germany) following the manufacturer's instructions.

2.3.3. *Primer design and optimization*

To amplify the target fragment, the first promoter, the first intron, and the two first exons of the *SPAG11* gene (total length = 1452 bp), a pair of primers was designed using Serialcloner software version 2.6.1 and NCBI website. The sequences of the designed primers are as follows: Forward primer = 5′CGGCTATAAAGGAAGCCCTCCCTGCCGCCC3′: Primer melting temperature (Tm) = 69.9 °C; Reverse primer = 5′GCCCCCACAGTCACCTCCCATCCACTCTTC3′; and Primer melting temperature (Tm) = 67.9 °C. The primers were obtained from 1 st Base Co., Malaysia, in lyophilized form.

With the aim of producing a stable system to amplify the targeted DNA without nonspecific reactions, OligoAnalyzer 3.1 software was used to analyze the primer parameters. Due to the high annealing temperature (71/72 °C), the annealing and extension steps were combined (mixed) as a one-step PCR amplification (Jin et al., 2014). The annealing temperature (Ta), PCR conditions, and reaction components were optimized as described in Tables 1 and 2. Electrophoresis of the amplicons was conducted in 0.8% agarose gels containing SYBR® safe DNA gel stain (Invitrogen, USA) in 1x TBA buffer. The components of the reaction were assembled without a DNA template to indicate any contamination during PCR. The electrophoresis power supply program was adjusted to supply 80 V, 400 milliamp, and 100 W for 60 min. The gels were visualized and documented using a gel documentation system (Vilber, Lourmat, France) using ultraviolet (UV) light. Supplementary Fig. 1 includes a gel image of the desired fragment of the *SPAG11* gene after final optimization.

Table 1
Reaction components to amplify a fragment of *SPAG11* gene including Promoter 1, Intron 1, Exons 1 and 2.

Components (reagents)	Final conc.	Per 30 µl reaction
ddH_2O	–	Up to 30 µl = 10.95
2X KAPA HiFi HotStart ReadyMix	1X	15 µl
10 µM Reverse primer	0.3 µM	0.9 µl
10 µM Forward primer	0.3 µM	0.9 µl
50 ng/µl Template DNA	3.75 ng/µl	2.25

ddH2O: double-distilled water; KAPA HiFi HotStart ReadyMix is an engineered DNA polymerase.

Table 2
Thermocycling parameters of the two-step PCR program for designed primers to amplify a fragment of *SPAG11* gene including Promoter 1, Intron 1, Exons 1 and 2.

Steps	Temperature(°C)	Time (min:sec)	Cycles
Initialization*	95	03:00	1
Denaturation	98	00:20	25
Annealing and Extension**	72	00:30	
Final extension	72	02:00	1
Final hold***	4	Indefinite	

The lid preheated at 100 °C.

* According to KAPA instructions, the heat activation (hot-start PCR) was required.

** Between two optimal annealing temperatures, 72 °C was selected for this PCR. The annealing temperatures are high and close to the extension temperature.

*** Used for short-time storage of the reaction, then the PCR program was designed to keep the block cool at 4 °C after the extension step.

2.3.4. *Molecular cloning and genotyping*

To clone the full length of the target *SPAG11* gene fragment, molecular cloning was conducted for all 52 samples using a Zero Blunt® TOPO® PCR Cloning Kit (Invitrogen, USA). The PCR products were purified using a FavorPrep™ MicroElute GEL Purification Kit (Favorgen Biotech Corporation, Taiwan) according to the manufacturer's instructions. The PCR cloning products were assessed using a 0.8% agarose gel to validate the use of the process accomplished what was intended (Supplementary Fig. 2). The selected clones were cultured in a tube containing LB broth and kanamycin and then incubated at 37 °C overnight. All tubes were centrifuged at 3500 RPM for 15 min at 4 °C to pellet a condensed plasmid. The plasmids were purified using Easy Pure Plasmid MiniPrep Kit (Quan-jin Biotech Co., Beijing, China). With the aim of single clone sequencing, several clones from each individual were cultured in separate tubes (containing LB broth and kanamycin). The SNP genotyping was conducted based on sequence electropherograms (Supplementary Figs. 3–6). The sequencing of purified plasmids was performed with Sanger sequencing using a vector-specific primer (rather than a PCR primer) through single-pass sequencing of independent purified plasmids in both directions (forward and reverse strand) by a sequencing company (1st Base Co., Malaysia).

2.3.5. *SNP investigation and statistical analyses*

The SNPs were detected using a single base extension (SBE) method. The target fragments of the *SPAG11* gene were investigated based on a reference sequence (accession number: NC_030834.1) available in NCBI website. The SNPs at these regions were identified through the sequences using a bioinformatics software (Geneious Pro, New Zealand) (Kearse et al., 2012).

Four SNPs were detected in the aforementioned exons, two of which were nonsynonymous in base *ss19199134543* (arginine to glutamine) and base *ss19199134541* (threonine to isoleucine). The others were synonymous in base *ss19199134540* and *ss19199134542*. The detected SNPs were coded as 0, 1, or 2 based on their genotypes. The normal distribution of dependent variables was tested using the Shapiro-Wilk test ($P < 0.05$), and outliers were identified using the scatter plots. A multiple linear regression model with three predictor variables was designed through the stepwise backward elimination method using a statistical software (JMP®, Version 11. SAS Institute Inc., Cary, NC, USA) Since, the bucks studied in this experiment were from a single herd and breed, the sire and interaction effect of sire by SNP were not effective on the outcomes of the mixed model. The final model, therefore, was designed without a sire effect. The following model (Eq. (1)) was considered to determine the association between detected SNPs and testes dimensions (length, width, and thickness), thickness of scrotum skin, TW, and TV using the levelled-container. The formula for this model is

$$Y = \beta_0 + \beta_1\chi_1 + \beta_2\chi_2 + \text{SNP} + \varepsilon \tag{1}$$

Where, *Y* is the observed traits value; χ_1, χ_2 stand for age of buck, scrotal circumference size, and *SNP* is the three levels factor (AA, AB, BB) while β_0 and ε are the intercept of the regression line and random errors, respectively.

2.3.6. *Estimating the additive and dominance effects of SNPs and haplotype analysis*

The additive effect (a) and dominance effect (d) of the SNPs with three genotypes and significant association with semen quality traits was estimated using Eqs. (1) and (3) (Lin et al., 2006) using an open-source programming languages (R, version 3.5.1, Austria) (R Core Team, 2019). The formulas of this model is

$$a = ½\,(BB – AA) \tag{2}$$

$$d = AB - ½\,(AA + BB) \tag{3}$$

where, AA, AB, and BB are indicated for least squares means of different genotypes of given traits. A *t*-test was used to examine the significance of additive and dominance effects ($\alpha = 0.05$). Eleven haplotypes of four SNPs were detected in the studied population.

Table 3
Estimated regression coefficients using a Passing-Bablok regression procedure.

	Estimate	Lower bound 95% CI	Upper bound 95% CI
Intercept	0.00	−3.09	12.50
Slope coefficient	1.00	0.96	1.01
Testing* $a = 0$ and $b = 1$	$P = 0.57$		

* Regression model is $y = a + bx$, and the P-value is testing for equality of methods ($y = x$).

3. Results

3.1. Levelled-container

There were no differences between TV values obtained with the levelled-container ($M = 268.57$, $SD = 96.48$) and the conventional equipment ($M = 266.17$, $SD = 96.00$) when assessed using a one-way ANOVA ($P = 0.45$). The estimated slope was 1.00 and the estimated intercept was 0.00. Thus, the results indicated complete agreement ($P = 0.57$) (Table 3). A scatterplot with a fitted regression line of the levelled-container and of the overflow vessel (Fig. 2), as well as the Bland-Altman plot indicated the data points were randomly distributed in the plots (Fig. 3).

3.2. SPAG11 association with TV

Four novel SNPs were identified in the coding region of the *SPAG11* gene (Supplementary Fig. 3–6). Four SNPs (*ss19199134542*, *ss19199134541*, *ss19199134540*, and *ss19199134543*) were detected in the studied coding region of the *SPAG11* gene (LOC106501767 CDS), two of which were synonymous (*ss19199134542* and *ss19199134540*) and other two were nonsynonymous SNPs (*ss19199134541* and *ss19199134543*). The SNPs *ss19199134541* and *ss19199134543* resulted in changes in the amino acids - Thr > Ile and Arg > Gln of the *SPAG11* protein, (protein ID: XP_017897296.1) respectively (Table 7).

The data for association between sperm-associated antigen 11 (*SPAG11*) gene polymorphisms and TV and testicular weight are included in Table 4. The detected SNPs had no effect on testicular weight, but the results indicate there is a marked negative association between SNP *ss19199134543* and TV ($P = 0.002$). The data for association analysis between testis dimension traits and detected SNPs are included in Table 5. None of the testicular dimensions were associated with detected SNPs. SNPs at *ss19199134543*, *ss19199134540* and *ss19199134542* loci were removed from the results that are reported due to the high standard error of the least square means for these loci for right length of testes. None of the calculated TVs were associated with *ss19199134543* SNP of SPAG11 gene (Table 8).

3.3. Additive and dominance effects and comparison of haplotype effects

The estimates of additive (a) and dominance (d) effects of SNPs on TV traits associated with *ss19199134543* genotypes are presented in Table 6. Comparisons of the least square means of TV for *SPAG11* haplotypes are depicted in Fig. 4 . The differences between TW haplotypes were not significant.

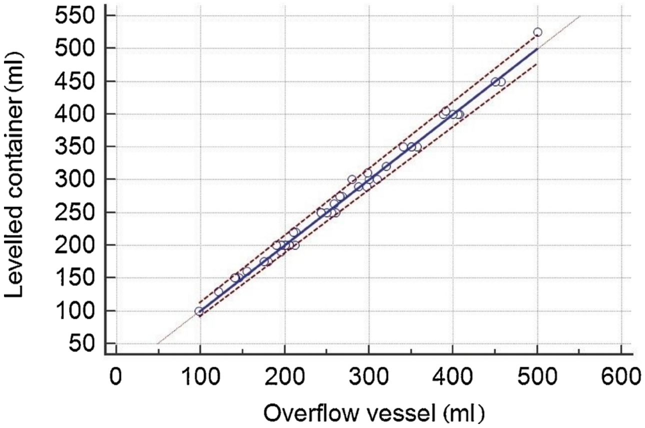

Fig. 2. Passing-Bablok regression plot of values obtained using the levelled-container and overflow vessel indicating the consistency in values obtained using the two methods ($n = 47$).

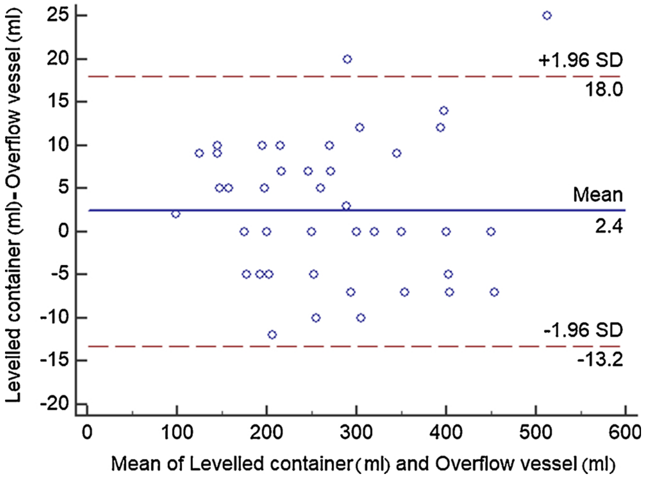

Fig. 3. Bland–Altman plots indicating the difference between testicular volumes obtained using the Overflow vessel (reference) and the levelled-container plotted against the mean when there was use of both types of equipment ($n = 47$).

Table 4
LS means (± SE) of testicular volume using Levelled-container and testicular weight indicating effects of detected SNPs of *SPAG11* gene.

SNPs position	Number of individuals	Testicular volume (ml)	Testicular Weight (g)
ss19199134543			
GG	35	292.45^{a} (8.05)	142.80^{a} (22.14)
AA	12	234.04^{b} (13.67)	77.66^{a} (38.16)
AG	4	249.01^{ab} (23.41)	74.38^{a} (65.28)
P-value		0.002	0.280
ss19199134540			
AA	49	274.28^{a} (7.45)	124.74^{a} (18.65)
GG	2	308.03^{a} (52.44)	93.51^{a} (51.75)
AG	0	–	–
P-value		0.527	0.486
ss19199134541			
CC	38	276.15^{a} (8.66)	128.62^{a} (21.42)
TT	10	276.86^{a} (16.69)	107.33^{a} (41.81)
CT	3	253.88^{a} (30.43)	88.86^{a} (76.25)
P-value		0.775	0.744
ss19199134542			
AA	49	275.26^{a} (7.56)	124.40^{a} (18.67)
GG	2	267.75^{a} (37.51)	93.58^{a} (66.07)
AG	0	–	–
P-value		0.845	0.544

[a,b]Values in a particular column (traits) with these differing superscripts were different ($P < 0.05$).

4. Discussion

The use of the two methods of measuring TV resulted in similar means (268.57 ml for the levelled-container and 266.17 ml for the conventional method) and were not different. Although the means when there was use of both devices were quite similar, the use of the overflow vessel required more time, attention, care and skill to read the volume accurately.

The estimated coefficient (Table 3) indicates that the slope interval (0.96–1.0177) and the intercept interval (−3.097–12.50) contain the values 1 and 0, respectively. The slope does not differ from zero and the intercept does not differ from 1. The use of levelled-container, therefore, is a valid method for measuring TV. These results are consistent with those from a study where there was another method of verification for use of this method in animals (Katsoulos et al., 2011). The results from use of the linear model validity test (Table 3, $P = 0.57$) indicate that there is sufficient evidence to accept the existence of linearity between measured TV using the levelled-container and the overflow vessel. These results indicate there is an association of data collected using the two methods. These results indicate that the use of levelled-container is a reliable method for measuring TV. Similar scatterplots for linearity have been reported by other researchers to assess the validity of new technology assessments (Kavsak et al., 2015). The random pattern in the Bland-Altman plot suggests that although two values were beyond the error limits, the mean difference was very close to zero. These results indicate that there was no bias in the estimates. There was neither a proportional bias, nor did the greater/lesser mean values tend to result in greater/lesser differences (Bland and Giavarina, 2015). These results were similar to

Table 5

LS means (± SE) of testes dimensions (length, width and thickness) and skin of scrotum diameter indicating the effects of detected SNPs of the *SPAG11* gene.

SNPs position	Number of individuals	Testes dimensions						
		Length of testes (cm)		Width of testis (cm)		Thickness of testes (cm)		Skin of scrotum diameter (cm)
		Right	Left	Right	Left	Right	left	
ss19199134543								
GG	35	8.79 (1.72)	5.62 (0.18)	3.87 (0.10)	3.83 (0.12)	3.42 (0.09)	3.32 (0.10)	0.57 (0.01)
GA	12	4.94 (2.97)	5.98 (0.32)	3.68 (0.17)	3.72 (0.20)	3.54 (0.17)	3.35 (0.18)	0.59 (0.03)
AA	4	–	5.39 (0.55)	3.87 (0.30)	3.94 (0.35)	3.67 (0.29)	3.37 (0.31)	0.55 (0.05)
P-value		0.448	0.542	0.642	0.827	0.680	0.985	0.761
ss19199134540								
AA	49	7.67 (1.44)	5.80 (0.12)	3.90 (0.06)	3.89 (0.06)	3.54 (0.06)	3.41 (0.07)	0.58 (0.01)
AG	2	–	5.50 (0.65)	3.72 (0.27)	3.77 (0.44)	3.68 (0.30)	2.89 (0.40)	0.55 (0.11)
GG	0	–	–	–	–	–	–	–
P-value		0.987	0.681	0.512	0.785	0.637	0.213	0.819
ss19199134541								
CC	38	8.01 (1.65)	5.68 (0.17)	3.77 (0.09)	3.81 (0.11)	3.44 (0.09)	3.31 (0.10)	0.58 (0.01)
CT	10	6.25 (3.22)	5.80 (0.34)	4.02 (0.19)	3.82 (0.22)	3.56 (0.18)	3.42 (0.19)	0.56 (0.03)
TT	3	–	5.31 (0.63)	3.89(0.34)	3.77 (0.40)	3.52 (0.33)	3.35 (0.36)	0.56(0.06)
P-value		0.840	0.795	0.513	0.993	0.846	0.888	0.800
ss19199134542								
AA	49	7.68 (1.44)	5.69 (0.15)	3.83 (0.08)	3.81 (0.09)	3.48 (0.08)	3.34 (0.08)	0.57 (0.01)
AG	2	–	5.57 (0.78)	3.73 (0.43)	3.94 (0.49)	3.27 (0.41)	3.26 (0.44)	0.66 (0.07)
GG	0	–	–	–	–	–	–	–
P-value		0.578	0.883	0.829	0.792	0.625	0.867	0.25

There were no difference between the studied testes dimensions/skin of scrotum diameter and different genotypes of the studied loci ($P \leq 0.05$).

Table 6

LS means (± SE) of estimated additive and dominance effects of the *ss19199134543* locus on testicular volume.

Locus effects	*ss19199134543*	
	Additive (*a*)	Dominance (*d*)
Testicular Volume	19.99^{ns} (12.74)	−35.67^{ns} (18.68)
P-Value	0.123	0.062

$^{*}P < 0.05$.
$^{**}P < 0.01$.
$^{***}P < 0.001$.
^{ns} = not significant.

Table 7

Summarized information about detected variants of *SPAG11* gene in Boar goats.

SPAG11 Loci	Based on chromosome 27 sequence	Based on SPAG11 gene sequence	Amino acid change	Type of mutation (SNP- transition)
ss19199134543	38364815C > T	1327 G > A	Arg > Gln†	Nonsynonymous
ss19199134540	38364817T > C	1325A > G	Gly > Gly††	Synonymous
ss19199134541	38364872G > A	1270C > T	Thr > Ile†††	Nonsynonymous
ss19199134542	38364886T > C	1256A > G	Gly > Gly††	Synonymous

SPAG11: Sperm-associated antigen 11 gene.
† Arginine > glutamine.
†† Glycine > Glycine.
††† Threonine > Isoleucine.

Table 8

LS means (± SE) of testicular volume using FA, FB and FC formulae indicating effects of the *ss19199134543* SNP of the *SPAG11* gene.

SNPs position	Number of individuals	TV- FA (ml)	TV- FB (ml)	TV- FC (ml)
ss19199134543				
GG	35	117.92 (20.02)	159.90 (27.15)	132.70 (20.94)
AA	12	71.50 (35.95)	96.95 (48.75)	75.03 (37.60)
AG	4	61.97 (59.29)	84.04 (80.39)	67.89 (62.89)
P-value		0.431	0.431	0.325

Testicular volume by Prolate ellipsoid shape formula (TV-FA).
Testicular volume by Lambord formula (Empirical shape) (TV-FB).
Testicular volume by Hansen-Bessel formula (Prolate spheroid shape) (TV-FC).
There were no difference between the calculated TVs using different formulae and different genotypes of the studied locus ($P \leq 0.05$).

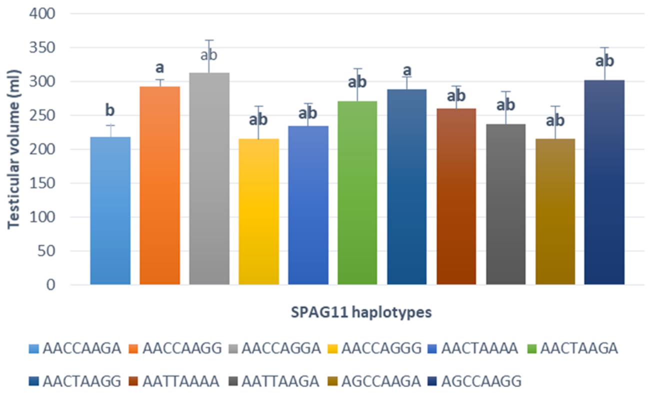

Fig. 4. Least squares mean comparison of the testicular volume trait for different *SPAG11* haplotypes; Different superscripts (^{a,b}) indicate differences ($P < 0.05$).

those previously reported (Katsoulos et al., 2011).

To measure an accurate TV using a levelled-container, the container should be maintained in a level position in both horizontal and vertical directions during measurements. This is possible with the help of two attached bubble levels on the designed equipment. The attached vial levels allow the operator to read the exact volume during the time that the bubbles of both vials are positioned on the center of the vials. The levelled-container may be constructed in different sizes by companies that produce animal reproduction equipment used for measuring TV in the other species. The novel method is beneficial as compared with the conventional method in that the use of the levelled-container is easier and there is requirement for a single piece of equipment. The amount of time required for collecting data is less than with the conventional equipment where there are two devices needed to collect water overflow using a graduated cylinder. Due to the beneficial value of measuring TV as a measure of fertility (Avdi et al., 2004), the modified beaker (levelled-container) approach is a practical approach for use in measuring the TV in the management and selection of bucks (Strina et al., 2016), even by rural farmers.

Overall, the results of the present study indicate that the use of the levelled-container is as effective an approach for measuring TV as the use of the overflow vessel. In addition, considering the ease of use of the levelled-container this approach to measuring TV can be easily implemented, which can lead to improved selection and management of livestock especially for sustainable goat production within the resource-poor farming regions. It is concluded that the evaluation of this technique in the present study provides for sufficient evidence to utilize the levelled-container as a user-friendly alternative for measuring TV in livestock.

In the present study there was investigation of the SPAG11 gene polymorphisms using single base extension (SBE) method for SNP discovery along with molecular cloning with the aid of the Sanger sequencing method for the first time in goats. The advantage of molecular cloning allows for the investigation of the full length of the desired fragment gene, it may be that the integration of molecular cloning along with a single base extension (SBE) method could be a reasonable strategy to discover de novo SNPs even in small populations. There was a strong association between SNP, *ss19199134543*, and TV, which can be explained in three possible ways. The primary reason for differences between two groups of bucks (with and without detected *ss19199134543*) may be due to structural differences in their testicular parenchyma density (Wu et al., 2010). Because of the relationship between the volume and weight of an object, there is an assumed relationship between the density of a specifc testes and the weight of the testicular tissue as well as that the volume is directly and inversely related, respectively (Mendis-Handagama and Ewing, 1990). Both testicular weight and volume, however, are directly related with SNP, *ss19199134543*. Thus, the variation in testis density may not be the reason for the results in the present study. Another possibility could be related to the variety of testes dimensions; in this study, however, the testes dimensions were not associated with *ss19199134543* (Table 5). Another theory is from an anatomical perspective. Indeed, TV measurements evaluated using the water displacement method are not the measurement of the actual TV. While measuring TV, the volume of some other anatomical attached components to testes (e.g., spermatic cord, vas deferens and layer of tunica vaginalis partly, and epididymis mostly) will be included in the estimate of TV. Thus, it is likely that the detected SNP, *ss19199134543*, affects the functions of these other tissues. Among these, the epididymis (head, body and tail) is one of the major components that can affect the TV. The epididymis has an important function in sperm development such as percentage of abnormal sperm and motility (Gloria et al., 2011). To confirm this theory in a practical way, the TV should be calculated without considering the other anatomical attached components by using testes dimensions and mathematical formulae. The association between the calculated TV and the ss19199134543 SNP should subsequently be investigated. The Eq. (1), therefore, was used to investigate the association between each TV−FA, TV−FB and TV−FC as the response variable (Y) and the *ss19199134543* SNP separately. Results indicated that none of the calculated TVs were associated with the desired SNP (Table 8). The significant differences among different genotypes of the ss19199134543 locus, therefore, was most likely a consequence of the SNP effects on volume of anatomical components that are attached to the testes (e.g., spermatic cord, vas deferens and layer of tunica vaginalis partly, and epididymis mostly). Significant associations between *ss19199134543* SNP and several semen quality variables in fresh and post-thaw semen have been reported (M.F. Harighi unpublished doctoral thesis, 2019), which is a logical reason for this consideration.

Due to marked effect of the *SPAG11* gene on the epididymis (Biswas and Yenugu, 2013; Ribeiro et al., 2012), it can be concluded that the most likely effect of the *ss19199134543* locus is on anatomical components that are attached to testes that resulted in a change in the estimated TV among different genotypes. Results of several studies indicate there are effects of the gonadotropin receptor gene polymorphism (Simoni et al., 2008) and heat shock protein 90 gene (Hassun Filho et al., 2005) on TV of humans.

The additive and dominance analysis in the present study indicated that the SNP, *ss19199134543*, tended to have a dominance effect on TV ($P = 0.062$). In addition, there was no ($P < 0.05$) difference between the mean of the TV for a heterozygote with a *ss19199134543* and the homozygote for this locus. This finding may indicate the partial dominance effect of alleles at locus *ss19199134543*. Comparison of the least square means of different haplotype effects indicated the marked effect of the *ss19199134543* locus on TV. The haplotype comparison results indicated that the goats with the AACCAAGG and AACTAAGG haplotypes had the largest TV, while bucks with the AACCAAGA haplotype had the smallest TV.

5. Conclusion

In conclusion, most likely, the ss19199134543 locus of the *SPAG11* gene has an effect on anatomical components that are attached to testes, causing changes in the TV. Water displacement, therefore, was found to be a more efficient method for measuring TV in comparison to use of mathematical methods. The TV is a testicular biometric variable that can be used for preselection of bucks with desirable semen quality. Further investigations are suggested to ascertain the optimal TV for fertile bucks based on semen quality variables at different age categories. Results of the present study indicate the levelled-container is a user-friendly alternative for measuring TV in livestock. The efficiency of use and precision of measurements with use of this device is sufficient evidence that can be taken into consideration by farm managers and other researchers in future studies.

Acknowledgments

This study was supported by a research grant from Universiti Putra Malaysia (UPM) [GP-IBT/2013/9418900]. The authors would like to express their gratitude to the Department of Veterinary Services-Malaysia for offering their farm to conduct this project.

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.anireprosci.2019.106113.

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