



## Seminal attributes of Vechur bulls (*Bos indicus*), the smallest humped dairy cattle

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### ABSTRACT

The study was undertaken at Mattupatti semen station to objectively evaluate the seminal attributes of frozen-thawed semen of Vechur bulls, the smallest humped dairy cattle in the world, using the computer assisted semen analyzer (CASA) and Flow cytometer. The mean motility and progressive motility of the frozen-thawed semen estimated via CASA was  $77.25 \pm 9.56$  and  $64.0 \pm 4.21\%$  respectively. Mean values obtained for the sperm kinetic parameters like path velocity (VAP), progressive velocity (VSL), track speed (VCL), lateral head amplitude (ALH), beat cross frequency (BCF), straightness (STR) and linearity (LIN) were  $82.56 \pm 12.12$ ,  $76.22 \pm 10.38$ ,  $107.77 \pm 21.42$ ,  $4.39 \pm 0.91$ ,  $20.87 \pm 3.81$ ,  $92.37 \pm 3.25$  and  $72.87 \pm 6.64\%$  respectively. Flow cytometry analysis of the functional sperm parameters like sperm viability, live sperms with intact acrosome, sperms with normal mitochondrial membrane potential (MMP) and sperms with altered DNA were  $81.67 \pm 4.55$ ,  $76.16 \pm 9.76$ ,  $82.40 \pm 5.61$  and  $11.75 \pm 0.3\%$  respectively. The mean value of the HOS reactive sperms was  $71.81 \pm 5.71\%$ . Mean values for the length and width of the sperm head, length of mid-piece, tail and overall sperm length measured by computer image analysis was  $8.99 \pm 0.36$ ,  $5.07 \pm 0.23$ ,  $14.21 \pm 0.35$ ,  $45.12 \pm 1.53$  and  $68.32 \pm 1.41 \mu\text{m}$  respectively. Study concluded that seminal attributes of Vechur bulls could be estimated with CASA and Flow cytometer and the parameters were comparable to other indigenous, crossbreds and exotic dairy cattle.

**Keywords:** CASA, Flow cytometer, Seminal attributes, Vechur bulls

Vechur cattle, an indigenous rare dairy breed of Kerala, are the smallest humped cattle in the world. Mean height and length of bulls are  $89.43 \pm 6.55$  cm and  $102.25 \pm 1.52$  cm respectively; and average body weight is around 170 kg. Despite its small size, average daily milk yield of the cow is  $2.5 \pm 0.5$  litres. Small size of the animal, minimal feed requirement, good adaptability and disease resistance are the favoured traits that promote dairy farmers to maintain Vechur cattle. The frozen semen station of Kerala Livestock Development Board (KLDB), located at Kulathupuzha in Kollam district, South Kerala maintains few Vechur bulls for semen production as a part of the breed conservation programme of the state. The station makes available quality semen of the breed to the entire state for artificial insemination.

The present study (first of its kind for Vechur) was undertaken to objectively evaluate the seminal attributes of Vechur semen, including the sperm biometry. Sperm motility and kinetic parameters like average path velocity (VAP), progressive velocity (VSL), track speed (VCL), lateral head amplitude (ALH), beat cross frequency (BCF), straightness (STR) and linearity (LIN) of semen was

evaluated using CASA. Flow cytometer was used for assessing functional sperm parameters like cell viability, live sperms with intact acrosome, mitochondrial membrane potential (MMP) and sperm chromatin structure assay (SCSA). The plasma membrane integrity of the sperm cells was assessed by the Hypo osmotic swelling (HOS) test. Further, the biometry of Vechur spermatozoa was estimated by means of Computer Image Analysis.

### MATERIALS AND METHODS

Frozen semen production of the Vechur bulls were undertaken at Kulathupuzha semen station. Semen was collected from eight clinically healthy and scientifically managed bulls using artificial vagina. Only ejaculates with an initial motility of 70% and above and with a sperm concentration above 500 million/ml qualified for processing. After evaluation, semen was extended using the Tris extender such that each French mini straw contained 20 million spermatozoa. After an equilibration period of 4–6 h, the semen straws were frozen using a Programmable Bio-freezer. The post thaw motility of the samples was examined after 24 h of freezing. Only samples with a minimum post-thaw motility of 50% and above qualified for field distribution. The semen straws were released for field distribution only after the quarantine period of thirty days.

After the quarantine period, 320 doses of frozen semen

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produced from eight Vechur bulls were procured from Kulathupuzha semen station for undertaking the study. Two batches of semen were randomly selected from each of the bull; each batch consisting of twenty straws each. The semen straws were transported to the Quality Control Laboratory of Mattupatti Semen Station in a liquid Nitrogen refrigerator, observing standard semen handling protocols. The study was undertaken during the period from January to February 2020.

Two semen straws of each bull (one straw from each batch) were thawed and examined for post-thaw motility and sperm kinetic parameters using the CASA, Hamilton Thorne Biosciences, USA at the recommended starting analysis settings of the equipment manufacturer.

Two semen straws of each bull (one straw from each batch) were thawed and used for estimating the seminal attributes like sperm cell viability, live sperms with intact acrosome, MMP and SCSA using the BD Accuri C6 Flow Cytometer, USA

HOS test of the frozen straws of the bulls of different batches were undertaken to assess the proportion of sperms with bio-chemically active plasma membrane as per standard procedure.

Two thawed semen straws of each bull (different batches) were stained using the Giemsa stain and then examined under the DIC Microscope (Olympus BX 51) at 100× magnification (oil immersion) for the analysis of the biometry of Vechur spermatozoa. The Microscope had an attached camera, interfaced to a computer and the Image-Pro Plus Version 6.0 software for image analysis. The software facilitates to take the linear measurement of each sperm, i.e. length and width of sperm head, length of mid piece, tail length and total length of spermatozoa. Only conspicuously stained spermatozoa exhibiting normal morphology were measured. Ten sperms were measured in each slide and a total of 160 sperms of the eight bulls were measured. The unit for measurement of sperm biometry was micrometers ( $\mu\text{m}$ ).

The values obtained for the various seminal attributes and sperm biometry were analyzed for mean and standard deviation.

## RESULTS AND DISCUSSIONS

The range and mean values of sperm motility and other kinetic parameters of frozen - thawed Vechur semen estimated via CASA are depicted in Table 1.

Motility facilitates the spermatozoa to reach the site of fertilization in female reproductive tract and is a vital parameter for high conception rates. In the study, motility ranged from 63.0 to 94.0% with a mean of  $77.25 \pm 9.56\%$ . The progressive motility ranged from 57.0 to 71.0% with a mean of  $64.0 \pm 4.21\%$ . The findings of this study were in accordance with Islam *et al.* (2017), who reported total motility of  $72.85 \pm 1.46\%$  and progressive motility of  $64.80 \pm 1.51\%$  in crossbred bulls. Higher values for progressive motility have been reported by Vincent *et al.* (2012) and lower values by Contria *et al.* (2010) in related studies.

Table 1. Range and mean  $\pm$  SD of the motility and sperm kinetic parameters of Vechur semen

| Motility and kinetic parameter                 | Range      | Mean $\pm$ SD      |
|------------------------------------------------|------------|--------------------|
| Motility (%)                                   | 63.0–94.0  | $77.25 \pm 9.56$   |
| Progressive motility (%)                       | 57.0–71.0  | $64.0 \pm 4.21$    |
| Rapid (%)                                      | 58.0–80.0  | $66.62 \pm 6.19$   |
| Medium (%)                                     | 3.0–27.0   | $10.75 \pm 7.85$   |
| Slow (%)                                       | 1.0–11.0   | $4.0 \pm 3.66$     |
| Static                                         | 4.0–36.0   | $18.75 \pm 11.04$  |
| Path velocity (VAP) ( $\mu\text{m/s}$ )        | 69.0–103.9 | $82.56 \pm 12.12$  |
| Progressive velocity (VSL) ( $\mu\text{m/s}$ ) | 64.0–90.9  | $76.22 \pm 10.38$  |
| Track speed (VCL) $\mu\text{m/s}$              | 87.4–151.7 | $107.77 \pm 21.42$ |
| Lateral head amplitude (ALH) $\mu\text{m/s}$   | 3.3–6.1    | $4.39 \pm 0.91$    |
| Beat cross frequency (BCF) Hz                  | 16.0–28.5  | $20.87 \pm 3.81$   |
| Straightness (STR %)                           | 87.0–96.0  | $92.37 \pm 3.25$   |
| Linearity (LIN %)                              | 62.0–84.0  | $72.87 \pm 6.64$   |

Total motile sperms and progressive motile sperms differed between studies owing to breed difference, seasonal effect, initial quality of harvested semen, semen handling and processing protocols followed and the type of equipment/ software used for analysis.

The mean values for rapid, medium and slow sperms were  $66.62 \pm 6.19$ ,  $10.75 \pm 7.85$  and  $4.0 \pm 3.66\%$  respectively. The static sperms were  $18.75 \pm 11.04\%$ . No literatures could be traced on the above parameters in related studies for comparison.

Mean values obtained for velocity parameters such as VAP, VSL and VCL were  $82.56 \pm 12.12$ ,  $76.22 \pm 10.38$  and  $107.77 \pm 21.42 \mu\text{m/s}$  respectively. The results of the study were in line with Sundararaman *et al.* (2012), who reported 90.5, 71.5 and  $152.1 \mu\text{m/s}$  respectively for VAP, VSL and VCL in a similar study. Sperm kinematic parameters such as VCL, VSL, VAP and LIN were reported to be positively correlated with bull fertility (Perumal *et al.* 2011). A close correlation (with high significance) between each of the individual velocities (VAP, VSL and VCL) and the non-return rates was reported by Nagy *et al.* 2015. Their study concluded that VAP values obtained from CASA should be preferred to evaluate and predict the fertilizing potential of fresh or frozen-thawed bull semen. In the study, the results for all velocity parameters (including VAP) obtained from CASA for the Vechur frozen- thawed semen were satisfactory, establishing that the frozen semen was appropriate for field insemination.

ALH ranged from 3.3 to 6.1 with a mean of  $4.39 \pm 0.91 \mu\text{m/s}$ . Slightly higher values were obtained for ALH by Amanada (2011) in Holstein bulls. The results in respect of BCF ranged from 16 to 28.5%, with a mean of  $20.87 \pm 3.81$  Hz. Similar results were also reported by Perumal *et al.* 2014 and Amanda (2011) in related studies. However, studies by Pathak *et al.* (2019) on Gir, Surti and Murrah bulls have observed lower values for both ALH and BCF as compared to this study. The variations in results of ALH and BCF between studies may be attributed to differences in breed, reproductive potential of bulls, quality of semen

harvested, processing protocol followed and the model of the CASA used.

In the study, STR ranged from 87.0 to 96.0% with a mean value of  $92.37 \pm 3.25\%$  and LIN from 62.0 to 84.0% with a mean of  $72.87 \pm 6.64\%$ . The STR values reported in the study of Pathak *et al.* (2019) was in close agreement with the present study. But their results for LIN was relatively lower (48.49 to 50.06%). Studies by Ferrel *et al.* (1998) had revealed that semen samples containing sperms with higher liner motility had higher conception rates following artificial insemination. The high LIN value of 72.87% obtained for Vechur in the study is suggestive of quality semen with high conception rate.

The range and mean values of the seminal attributes of frozen - thawed Vechur semen estimated with the Flow cytometer are illustrated in Table 2.

Viability of spermatozoa is a key determinant of semen quality and pre-requisite for successful fertilization. The mean sperm viability was  $81.67 \pm 4.55\%$ . The results of the study are in close agreement with Garcia Macias *et al.* 2007, who reported  $59.9 \pm 1.9$  and  $76.8 \pm 2.5\%$  viable sperms in crossbred and purebred bulls respectively. The mean value of the dead sperms was  $18.33 \pm 4.55\%$  in the study.

Spermatozoa must maintain an intact acrosome up to the time it binds to the zona pellucida of the oocyte and undergo acrosome reaction. Hence, a high proportion of live sperms with functionally intact acrosome should be available in the semen straws for attaining high conception rates. Live sperms with intact acrosome varied from 73.98 to 81.68% with a mean value of  $76.16 \pm 9.76\%$  in the study. The results of the study agreed with the findings of Garcia Macias *et al.* (2007) and Zodinsanga *et al.* (2015). Live sperms with reacted acrosome was only  $0.23 \pm 0.32\%$ , illustrating that the Vechur spermatozoa could effectively circumvent the freezing injury. Kumaresan *et al.* (2001) had reported a high correlation between fertility and percentage of sperms with normal acrosome. The proportion of live sperms with intact acrosome observed in the study is illustrative of good quality semen. The dead sperms with

intact acrosome and reacted acrosome were  $11.27 \pm 10.07$  and  $5.35 \pm 1.18\%$  respectively.

Mitochondria are the major organelles for the production of adenosine triphosphate (ATP) which is important for maintaining flagellar movement and required for sperms to reach the site of fertilization. So, measurement of MMP provides information about the fertility status of the sperms indirectly. The reduction in fertility of frozen-thawed semen could be attributed to changes in temperature during the freezing process as well as osmotic and toxic stress owing to exposure to the cryoprotectant and formation of ice crystals (Papa *et al.* 2015). In the study, the sperms with normal MMP ranged from 74.13 to 90.21 with a mean of  $82.40 \pm 5.61$  and sperms with abnormal MMP ranged from 9.79 to 25.87 with a mean value of  $17.60 \pm 5.60$ . The findings of the study were in close agreement with Ajaoa *et al.* (2015) and Krishnan *et al.* (2016) in related studies. Spermatozoa that exhibit high MMP, generally have intact acrosome function and high fertilizing capacity as well as normal motility and morphology (Grunewald *et al.* 2008). In the study on Vechur, live sperms with intact acrosome and sperms with normal MMP were on the satisfactory limit.

Spermatozoa with abnormal DNA condensation are found in low numbers in semen of normal fertile bulls. But the incidence increases, following disturbances in spermatogenesis. Sperms with altered sperm chromatin structure ranged from 11.03 to 12.18, with a mean of  $11.75 \pm 0.35\%$  in Vechur semen. The findings of the study are in line with Dobrinski *et al.* (1994), who reported mean value of  $9.8 \pm 1.1$  for altered sperm DNA. Higher values (1.2 to 23.8%) of altered chromatin structure were reported by Bochenek *et al.* (2001), who observed a high variation in sperm chromatin structure between different ejaculates within bulls of low fertility rates. Evaluation of the sperm chromatin structure has been reported to be highly correlated to fertility in cattle (Ballachey *et al.* 1988). SCSA may hence be useful in identifying bulls of suboptimal fertility in breeding soundness evaluation of newly inducted bulls and also be used for monitoring the semen quality of bulls under regular production.

The HOS reactive sperms in Vechur semen ranged from 68.0 to 79.5% with a mean of  $71.81 \pm 5.71\%$ . It was observed that more than 50% of the sperms showed swelling of the entire tail region. Lodhi *et al.* (2008) had reported higher HOS positive sperms (85.25%) in their study on fresh Sahiwal and Nili Ravi semen. But Pathak *et al.* (2018) had reported lower mean values for HOS reactive sperms in their studies ( $48.25 \pm 0.78$ ,  $44.21 \pm 1.29$  and  $51.54 \pm 1.29\%$  for Gir, Surti and Murrah bulls respectively). The percentage of HOS reactive sperms differs between studies on account of bull variations, strength/ concentration of test solution used, semen quality, live sperms with intact acrosome and individual fertility levels. The present study revealed that a high proportion of Vechur sperms had an intact and biochemically active plasma membrane capable of fertilizing the ovum.

Using "Image-Pro plus version 6.0" software,

Table 2. Range and Mean $\pm$ SD of the seminal attributes of Vechur semen

| Seminal attribute (%)                   | Range       | Mean $\pm$ SD     |
|-----------------------------------------|-------------|-------------------|
| Sperm cell viability                    | 75.36–87.53 | $81.67 \pm 4.55$  |
| Dead sperms                             | 12.47–24.64 | $18.33 \pm 4.55$  |
| Live sperms with intact Acrosome (LIA)  | 73.98–81.68 | $76.16 \pm 9.76$  |
| Live sperms with reacted Acrosome       | 0.02–0.89   | $0.23 \pm 0.32$   |
| Dead sperms with intact Acrosome        | 0.79–30.44  | $11.27 \pm 10.07$ |
| Dead sperms with reacted Acrosome       | 3.77–7.49   | $5.35 \pm 1.18$   |
| Sperms with normal MMP                  | 74.13–90.21 | $82.40 \pm 5.61$  |
| Sperms with abnormal MMP                | 9.79–25.87  | $17.60 \pm 5.60$  |
| Sperms with altered chromatin structure | 11.03–12.18 | $11.75 \pm 0.35$  |



Table 3. Range and Mean  $\pm$  SD of the biometric measurements of Vechur spermatozoa

| Parameter                          | Range       | Mean $\pm$ SD    |
|------------------------------------|-------------|------------------|
| Length of sperm head (mm)          | 7.93–9.67   | 8.99 $\pm$ 0.36  |
| Width of sperm head (mm)           | 4.60–5.58   | 5.07 $\pm$ 0.23  |
| Length of sperm mid-piece (mm)     | 13.62–14.78 | 14.21 $\pm$ 0.35 |
| Length of sperm tail (mm)          | 42.49–47.31 | 45.12 $\pm$ 1.53 |
| Overall length of spermatozoa (mm) | 65.94–71.03 | 68.32 $\pm$ 1.41 |

conspicuous (well stained) and morphologically normal sperms were measured and the values are illustrated in Table 3.

The mean values for the length and width of sperm head, length of mid-piece, length of tail and overall length of sperms were 8.99 $\pm$ 0.36, 5.07 $\pm$ 0.23, 14.21 $\pm$ 0.35, 45.12 $\pm$ 1.53 and 68.32 $\pm$ 1.41mm respectively. The values of Standard Deviation (SD) in respect of the different biometric parameters were significantly low in the study indicating the exactitude of the measuring software/ tool. Shahani *et al.* (2010), studying the relationship between mid-piece length of sperm and fertility in different cattle breeds has reported sperm head length, its width, and mitochondrial length as 14.25 $\pm$ 0.17, 7.27 $\pm$ 0.23 and 20.30 $\pm$ 0.15 mm respectively. Variations noticed in the sperm measurements between studies may be due to breed difference and the technique used for recording the measurements.

The study concluded that assessment of the seminal attributes of frozen-thawed Vechur semen via Computer assisted semen analyzer and Flow cytometer would facilitate to comprehensively investigate the reproductive potential of Vechur bulls and provide a reasonable forecast of semen fertility. A combination of investigations/ quality checks would be worthwhile for predicting fertility. The aforementioned equipments could be effectively utilized for monitoring semen quality in sperm production stations and also assist the Breeding Soundness Evaluation (BSE) of bulls. Further, the study also disclosed that the seminal attributes of Vechur, the unique dwarf dairy cattle of Kerala were comparable to other indigenous, crossbreds and exotic dairy cattle.

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