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ORIGINAL ARTICLE

Quality of frozen semen of Brahman bulls used for routine artificial insemination in Bangladesh

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Abstract

Background: Semen must be of good quality to achieve satisfactory conception rate in any artificial insemination (AI) programme. The objectives of the present study were to evaluate the quality of frozen semen of Brahman bulls and compare the same semen derived from different sources.

Methods: Fifteen frozen semen straws of Brahman bulls derived from 3 different sources (5 straws from each source) were evaluated with respect to volume, motility, concentration of spermatozoa and morphology of spermatozoa. Volume of semen was determined by micropipette, concentration was evaluated by haemocytometer technique, motility and progressive motility were evaluated by computer assisted sperm analyser (CASA), spermatozoa with normal acrosome, midpiece and tail were evaluated in formol-saline fixed semen and spermatozoa with normal head morphology were evaluated by Farley staining technique. Moreover, presence or absence of bacteria in semen was evaluated by Gram's staining technique.

Results: The overall semen volume, concentration, motile percentage, progressive motile percentage, percentage of spermatozoa with normal acrosome, midpiece and tail, and percentage of normal head morphology were 0.19±0.01 ml, 30.87±3.4x10^6/straw, 56.47±24.2 (%), 16.23±9.8 (%), 88.23±2.1 (%) and 95.1±1.6 (%), respectively. When compared, similar volume of semen (0.19 ml) was found in all frozen semen derived from different sources. The difference in percentage of motile spermatozoa between source B and C was significant ($P<0.05$). The difference in spermatozoa with normal acrosome, midpiece and tail in semen between source A and B was significant ($P<0.05$). The difference in the percentage of progressive motile spermatozoa, the concentration of spermatozoa and normal head morphology in semen among different sources of semen was not significant ($P>0.05$). The difference in percentage of motile spermatozoa, the percentage of progressive motile spermatozoa, the concentration of spermatozoa, normal acrosome, midpiece and tail, and normal head morphology in semen between locally produced and foreign semen was not significant ($P>0.05$). All examined 15 semen samples were negative for presence of bacteria.

Conclusions: Although the quality of frozen semen derived from Brahman bulls seems to be within normal limit, the post-AI conception rate should be determined to evaluate the fertilizing capacity of the used semen.

Key words: Artificial insemination, Brahman bulls, frozen semen, quality

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Introduction

About 85% of cattle of Bangladesh are of non-descriptive and indigenous in origin with low productivity compared to other exotic breeds and their crosses (Hamid *et al.*, 2017). For upgradation of our local breed, artificial insemination (AI) has been in use since 1960s as it is regarded as an important tool for crossbreeding throughout the world. Selection of disease free bull including ensuring quality semen is very important for an effective AI programme. The post-AI fertilizing ability of spermatozoa depends not only on the initial quality of semen, but also on the subsequent laboratory processes that end up with deposition of semen in the genital tract of a cow (Kunbhar *et al.*, 2019).The methods of semen dilution, chilling, freezing, storage, transportation and thawing for insemination can cause some reduction of the viability and fertilizing capacity of spermatozoa. Thus, the fertilizing capability of spermatozoa in the AI dose is determined not only by the inherent quality of the material produced by the bull but also by man's interaction with the product (Saacke, 1983; Serres *et al.*, 1997). Moreover, the process of transportation from one tank to another can warm up the semen straws if it is not accomplished quickly and accurately (Hafez, 1993). Maintaining optimum liquid nitrogen level in the container is important during prolonged storage and transportation of frozen semen (Sherman, 1990; Quintin *et al.*, 1997).

There is no specific beef breed of cattle in Bangladesh although there is presence of limited population of crossbred dairy cattle. Considering weather, agro-climatic condition, heat tolerance, disease and parasite resistance, longevity, grazing ability, calving ease, mothering ability and management, Brahman breed is considered to be the most suitable and compatible beef breed in tropical and sub-tropical regions (Antonio *et al.*, 2006). Recently, the Government of Bangladesh has introduced Brahman as beef breed to boost meat production through cross breeding of local cows with imported semen (Haque *et al.*, 2012). This has generated increased demand of good quality frozen semen of Brahman bulls in the country. However, the importation of semen is not without risk of carrying exotic diseases (Robert, 1986). So, our dependency on importing semen for routine use should be overcome by producing good quality frozen semen within the country. By this time, a small population of Brahman crossbred cattle has been produced and farmers are showing interest to rear these beef cattle. However, without having evaluation of quality of semen, it will not be wise to use Brahman semen widely in Bangladesh. Furthermore, the quality of native Brahman bull semen in contrast to imported ones has not yet been evaluated critically. Therefore, it is rationale to assess the quality of frozen semen of Brahman bulls to be used for routine AI in Bangladesh.

Hence, the objectives of this study were to determine the quality of commercial frozen semen of Brahman bulls used in selected areas in Bangladesh and to compare the quality among different sources of frozen semen and in between the locally produced and foreign frozen semen derived from Brahman bulls.

Materials and Methods

Study area

The study was conducted in Laboratory of Research Animal Farm (RAF) and Laboratory of Community-based Dairy Veterinary Foundation (CDVF), Department of Surgery and Obstetric, Bangladesh Agricultural University, Mymensingh.

Collection of semen straws

The frozen semen in 0.25ml French straws were collected from 3 different manufacturers including 5 straws from native source A, 5 straws from Foreign source B and 5 straws from Foreign source C. All semen straws were collected, transported and stored in liquid nitrogen container at -196°C until evaluation. Both native and imported semen were belonged to the national AI programme.

Evaluation of semen

Thawing of semen straws

The individual straws of semen were thawed by immersion into a water bath at 35-36˚C for 12 seconds.

Determination of semen volume and concentration of spermatozoa

Semen volume in eppendorf tube was determined by using micropipette and recorded. The concentration of spermatozoa (million/straw) was determined by using haemocytometer techniques. Briefly, semen samples were diluted with distilled water to kill the spermatozoa and the dilution ratio was 1:20 (semen: water). The Neubaur's counting chamber was focused under microscope first under low magnification (10x) and then under medium magnification (40x) objectives. A coverslip was placed on the counting chamber of haemocytometer. A very small drop of semen was transferred on Neubaur´s counting chamber. After 2-3 minutes, the number of sperm cells was counted in left top, right top, right bottom, left bottom and central secondary squares. Calculation of sperm concentration was performed following standard method.

Computer assisted sperm analysis (CASA)

Sperm kinematic was assessed objectively by using a CASA system consisting of a phase-contrast microscope. An aliquot (5 µL) of semen was deposited on a warmed microscope slide at 38°C and covered with a coverslip (18 × 18 mm). Sperm images in 8 fields were digitized for analysis of the kinematic patterns. The mean values were calculated for each of the following parameters based on approximately 1000 spermatozoa: total motility (TM%), progressive motility (PM %), VCL (velocity curved line, µm/s), VSL (velocity straight line, µm/s), VAP (velocity average path, µm/s), ALH (amplitude of lateral head displacement, µm), BCF (beat cross frequency, Hz) and the ratios STR (straightness, VSL/VAP), LIN (linearity, VSL/VCL), and WOB (wobble, VAP/VCL). Among these values total motility (TM%) and progressive motility (PM%) values were recorded for analysis of data.

Preparation of buffered formol-saline

Buffered formol-saline was prepared by dissolving disodium hydrogen phosphate with two molecules water (34.7mmol), potassium dihydrogen phosphate (18.7mmol), sodium chloride (92.6mmol) and formaldehyde (1.54mol) in distilled water.

Examination of acrosome, midpiece and tail of spermatozoa

The semen and buffered formol-saline were mixed at the same temperature to prevent any temperature variation-related damage of the spermatozoa. The abnormalities found in formol saline-fixed spermatozoa at phase contrast microscopy were classified as detached head, abnormal acrosome, proximal and distal cytoplasmic droplets, abnormal midpiece, double folded tail, bent tail, broken midpiece and coiled tail. At least 200 spermatozoa from individual replicates were examined at a magnification of 1000x.

Examination of head morphology

Thin smear was prepared from each of the semen sample for staining purpose. The morphology of sperm head was evaluated after staining with Farley stain. The Kit of Farley stain consisted of 3 reagents namely 3.5% formalin (Stain A), aniline-blue (Stain B) and crystal violet (Stain C). The smear was dipped in stain A for 10 seconds and in stain B for 20 seconds and washed by dipping into tap water. After that the slide again dipped in stain C for 5 seconds and washed by dipping into tap water. The slide was dried off and examined under compound microscope using 100x objectives. The head abnormalities in spermatozoa were classified as pear shaped, under developed head, double head, narrow head, broad head, abaxial position of the tail with the head and abnormal contour. At least 500 spermatozoa from individual smears were examined.

Gram staining technique

Gram staining was done in laboratory to detect the presence of microorganisms in a semen sample. Briefly, the semen smears were air-dried and then heat fixed. The smears were then gently flooded with crystal violet and let stand for 1 minute and then gently rinsed with tap water. The smears were gently flooded with Gram's iodine and let stand for 1 minute and again rinsed with tap water. Decolorization was done by using 95%

ethyl alcohol or acetone. The slides were tilt slightly and the alcohol was applied drop by drop for 5 to 10 seconds until the alcohol runs almost clear. Then immediately the slide was rinsed with water. The smears were gently flooded with Safranin to counter-stain for 45 seconds and rinsed with tap water. The slides were dried with bibulous paper. The smears were observed by using a light microscope under 100x objectives.

Statistical analysis

Analysis of variance followed by least significant difference test was done to find out significant difference in different parameters of semen among different manufacturers. Independent samples t-test was also done to find out the significant difference between locally produced and imported semen. The difference in semen parameters was considered significant when P value was less than 0.05.

Results

In the present study, irrespective of semen source, semen volume, concentration, motile percentage, progressive motile percentage, percentage of spermatozoa with normal acrosome, midpiece and tail, and percentage of normal head morphology were 0.19±0.01 ml, 30.87±3.4x10^6/straw, 56.47±24.2 (%), 16.23±9.8 (%), 88.23±2.1 (%) and 95.1±1.6 (%), respectively.

The data on quality of frozen semen of Brahman bulls with respect to different sources are presented in Table 1. Similar volume of semen (0.19 ml) was found in all frozen semen straws derived from different sources. The motile percentage of spermatozoa derived from native source A, foreign source B and foreign source C were 51.78, 46.28 and 71.34 (%), respectively. However, the difference in motile percentage of spermatozoa in frozen semen between foreign source C and foreign source B was significant (P<0.05). The progressive motile percentage of spermatozoa was numerically highest (20.3%) in frozen semen derived from foreign source C and the lowest (10.94%) was in semen derived from foreign source B. However, the difference in progressive motile percentage among different sources was not significant (P>0.05). The concentration of spermatozoa in frozen semen derived from different sources ranged from 30.60 to 31.6x10^6/straw. However, the difference in sperm concentration among different sources was not significant (P>0.05). The spermatozoa with normal acrosome, midpiece and tail in frozen semen derived from native source A, foreign source B and foreign source C were 89.2, 86.2 and 88.1%, respectively. However, the difference in spermatozoa with normal acrosome, midpiece and tail in frozen semen between source A and B was significant (P<0.05).The spermatozoa with normal head morphology in frozen semen derived from different sources ranged from 93.80 to 96.16%. However, the difference in proportion of spermatozoa with normal head morphology among different sources was not significant (P>0.05).

Table 1. Quality of frozen semen of Brahman bulls derived from different sources

Sources of semen	Volume (ml)	Motile percentage (%)	Progressive motile percentage (%)	Concentr-ation (10^6/straw)	Normal acrosome, midpiece and tail morphology (%)	Normal head morphology (%)
A (Native)	0.19±0.01	51.78±17.8^{ab}	20.30±13.0	30.60±2.4	89.20 ±1.9^{a}	95.32±1.1
B (Foreign)	0.19±0.01	46.28±13.8^{b}	10.94±9.1	30.40±4.2	86.20±2.2^{b}	93.80±0.8
C (Foreign)	0.19±0.01	71.34±6.5^{a}	17.44±2.2	31.60±2.5	88.10 ±1.3^{ab}	96.16±0.6

Values are Means ± SD; 5 straws from individual companies were examined.

^{a,b} Mean values with different superscripts in the same column differ significantly (p<0.05).

The data on comparison of the quality between foreign (Source B and C) and locally (Source A) produced frozen semen derived from Brahman bulls are presented in Table 2. Similar volume of semen (0.19 ml) was found in all frozen semen straws derived from locally produced and foreign sources. The motile percentage of spermatozoa derived from locally produced and foreign sources were 51.78 and 58.8 (%), respectively. The progressive motile percentage of spermatozoa derived from locally produced and foreign sources were 20.30 and 14.19 (%),

respectively. The concentration of spermatozoa in frozen semen derived from locally produced and foreign sources were 30.60 and 29.60x10^6/ml, respectively. The spermatozoa with normal acrosome, midpiece and tail in frozen semen derived from locally produced and foreign sources were 89.20 and 87.80%, respectively. The spermatozoa with normal head morphology in frozen semen derived from locally produced and foreign sources were 95.32 and 94.26%, respectively. However, the difference in semen parameters between locally produced and foreign semen was not significant (P>0.05).

Table 2. Comparison of the quality between foreign and locally produced frozen semen derived from Brahman bulls

Semen parameters	Locally produced	Foreign
Volume(ml)	0.19±0.01	0.19±0.01
Motile percentage (%)	51.78±17.8	58.80±16.6
Progressive motile percentage (%)	20.30±13.1	14.19±7.1
Concentration (10^6/straw)	30.60±2.4	29.60±4.1
Normal acrosome, midpiece and tail morphology (%)	89.20 ±1.9	87.80±2.4
Normal head morphology (%)	95.32±1.1	94.26±1.7

Values are Means ± SD; 5 straws from locally produced and 10 straws of foreign frozen semen were examined. Values in the same row did not differ significantly from each other (p>0.05).

When samples from 15 frozen semen straws were stained with Gram's staining technique, no bacteria were found in stained sample.

Discussion

The objective of the present study was to evaluate the quality of frozen semen of Brahman bulls routinely used in selected areas of Bangladesh. Moreover, the quality of frozen semen derived from different sources was compared. In the present study, irrespective of semen source, semen volume, percentage of motile spermatozoa, concentration, percentage of spermatozoa with normal acrosome, midpiece and tail and percentage of normal head morphology were within normal range. Similar sperm motility was reported by earlier study when frozen semen of Holstein-Friesian bulls was evaluated by CASA machine (Cojkic *et al.*, 2017). Contrasting to the present finding, the sperm motility after thawing observed in this study was numerically lower (56%) than that reported by Sundararaman *et al.* (2012) (63%) where spermatozoa of Jersey bulls were evaluated by CASA machine. Moreover, progressive sperm motility recorded in this study was lower (16%) than that of earlier studies in different breed of bulls (Sundararaman *et al.*, 2012; Cojkic *et al.*, 2017). The differences in motility of semen may be explained by the fact that the variation in the age of bulls, genetics, temperature and season of semen collection, feeding and semen collecting frequency between studies (Ismaya, 2014). Moreover, the variation in the quality of preserved frozen semen may be due to man-made manipulation during processing after its collection resulting in changes in sperm cell structures followed by poor sperm survival rate and fertilization capacity (Knox and Yantis, 2014). The proportion of spermatozoa with normal acrosome, midpiece and tail and with normal head morphology recorded in this study was 88.23±2.1 and 95.1±1.6%, respectively. This indicates that the abnormalities in spermatozoa were within normal limit as 20% sperm abnormalities are allowed according to Ax *et al.* (2000) since more than 20% sperm abnormalities may cause low fertility. Additionally, Balls and Peters (2004) demonstrated that a bull with more than 17% sperm abnormalities wouldn't have a high fertilizing capacity. In the present study, similar volume of semen was found in all frozen semen straws derived from different sources. This can be explained by the fact that all manufacturers used 0.25 ml French straws which were mostly filled in by automatic filling–sealing machine. Moreover, during checking quality of processed straws, the broken or half empty straws are discarded.

In the present study, percentage of motile spermatozoa significantly varied between semen of Brahman bulls derived from two foreign sources. Moreover, proportion of spermatozoa with normal acrosome, midpiece and tail in frozen semen significantly varied between native and one foreign source. This can be explained by the fact that the difference in post collection processing protocol and extender used between two studies may cause variation in quality of preserved semen. Moreover, fluctuation in temperature during storage and transportation may affect the quality of semen negatively. For successful cryopreservation, appropriate selection of extender including proper cryoprotective agent is essential that works well with the chosen extender to maintain high sperm motility (Varisli *et al.*, 2009). In the presence of a cryoprotective agent, the freezing behavior of the cells can be altered and glycerol is the most widely used cryoprotectant of semen (Holt, 2000; Barbas and Mascarenhas, 2009). Moreover, the post-thaw survival rate of spermatozoa was high when glycerol was added at +4 to 5°C and step wise mixture of glycerol with semen significantly improved the post-thaw survival rate of spermatozoa (Pickett and Berndtson, 1978).

For frozen preservation of semen, tris is superior to citrate/lactose/skim milk with regard to post-thaw motility and viability (Fath-el-bab and Yassen, l995). However, contrasting data also showed that both tris and milk-based diluents equally good with regard to post-thaw motility and fertilizing capacity of spermatozoa (Dhami and Sahni, 1993). Nevertheless, the extender and freezing protocol used for processing foreign semen of Brahman bulls are not known to the investigators. However, it is likely that well tested extender including standard freezing protocol was used for preservation of semen evaluated in the present study. Frozen semen can be stored successfully for years if stored continuously under liquid nitrogen at -196°C (Foote, 1978) and maintaining optimum nitrogen level in the container is important during prolonged storage and transportation of semen (Sherman, 1990; Quintin *et al.*, 1997). The first risk of sperm damage due to changes in temperature occurs during transfer of frozen semen straws from one container to other for delivery of semen to different centres and sub-centres. There are indications of significant reduction in post-thaw motility after one minute exposure of frozen semen straws to ambient temperature (Berndtson *et al.*, 1976).

In the present study, when compared, semen quality related parameters of Brahman bulls did not vary significantly between locally produced and foreign source. Contrasting to the present findings, sperm motility and proportion of spermatozoa with normal acrosome, midpiece and tail were significantly higher in imported semen than that of native counterpart in earlier study (Bhuiyan and Shamsuddin, 1999). The variation in semen parameters between two studies may be due to using different breeds of bulls (Brahman vs. Holstein-Friesian) and techniques of semen evaluation (Phase contrast microscopy vs. CASA). Moreover, semen handling during storage, distribution and transportation may influence the quality of preserved semen (Sugulle *et al.*, 2006). When samples from 15 frozen semen straws were stained with Gram's staining technique in the present investigation, no bacteria were found in stained sample. Absence of bacteria in stained semen sample indicates that the semen was processed under good hygienic conditions which are highly desirable. However, good measures should be taken by the inseminators during insemination to prevent bacterial contamination in AI gun followed by uterine infection and conception failure in inseminated cows.

Conclusions

The quality of frozen semen of Brahman bulls seems to be within normal limit. Further study is needed to determine the post-AI conception rate for evaluation of fertilizing capacity of the tested semen.

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Conflict of Interest

The authors declare no conflict of interest.

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