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## FACT FINDINGS ABOUT THE FROZEN BUCK SEMEN CHARACTERS FREEZING WITH EYC AND TRIS EXTENDER AND PRODUCTIVITY OF BLACK BENGAL DOES AS THE POTENTIAL GENETIC RESOURCE IN BANGLADESH

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### ABSTRACT

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The research work was conducted at the Artificial Insemination Center under the Department of Animal Breeding and Genetics, Bangladesh Agricultural University, Mymensingh to compare the efficacy between Egg Yolk Citrate (EYC) and Tris diluter for frozen semen production in Black Bengal buck. The parameters of semen characteristics included volume per ejaculate (ml), percentage of individual motility (progressive), normal and live sperm and sperm concentration (billion/ml). After dilution with EYC extender, It revealed from statistical analysis of frozen semen that individual bucks had significant effect ( $p < 0.05$ ) on sperm motility and but insignificant on motility after cooling with and without glycerol. In the same way, with Tris diluter, it was insignificant ( $p > 0.05$ ) on diluted semen motility and motility after cooling with and without glycerol. Motility and morphology of the sperm after equilibration and thawing showed insignificant difference among the bucks using EYC diluter. On the contrary, variation in the motility after equilibration and thawing was found significant ( $p < 0.05$ ) using Tris diluter and insignificant on normal and live sperm percentages. After insemination with frozen buck semen, productivity or conception rate was found significantly ( $p < 0.01$ ) higher (60.37%) in Tris than that of EYC diluter (43.75%). On the other hand, the productivity when compared among bucks within the diluter, the variation was not found significant ( $p > 0.05$ ). The productivity found both in Tris and EYC diluter is almost similar to abundantly used Triladyl diluter (58.25). It is concluded that tris diluter might be used as the alternative to the Tryladil diluter though further study is to be needed for more confirmation.

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## INTRODUCTION

Various factors like breeding soundness of male, semen quality sexual health of female, suitable diluters used etc. among others, play an important role in an AI program. Same buck has been used generation after generation which has created greater chance of increasing inbreeding depression and hence lowering reproductive performances along with disseminating of various venereal and infectious diseases in Black Bengal goat (Husain, 2007). Now it is well established that the selection of good quality bucks and their widespread use could improve the overall production potential of goats (Husain, 2007). To conduct frozen semen production, selection of suitable diluter is essential to test the efficacy. Considering the little work on this topic, this research has been taken to select suitable and economic diluter for frozen semen production taking into account the semen attributes. A number of diluents are used for buck semen preservation. Amiri (1997) found that Tris-Fructose-Egg-Yolk (TFEY) and Glucose-Citrate-Egg-Yolk (GCEY) are better diluents than skim milk. After a long experiment evaluating the different parameter of extender used determines the success of cryopreservation. Therefore, it seems rationale to compare the cryo-survival of spermatozoa considering characters with different diluters in the preservation of buck semen. Therefore, the development of suitable extender for preserving buck semen is essential for adoption of AI in goat. An extender should not only increase the volume of semen but also be capable of prolonging the life span of spermatozoa without compromising with the productivity. Suitable extender also determines the prolificacy of black Bengal does. It is also stable, economic and easy to prepare.

## MATERIALS AND METHODS

### Selection of breeding bucks and management

The study was conducted at the Artificial Insemination (AI) Center under the Department of Animal Breeding and Genetics, Bangladesh Agricultural University, Mymensingh. A total of six adult Black Bengal bucks were selected from Nucleus breeding flock (NBF) based on body weight, libido, reaction time, scrotal circumference (SC), volume and also their ability to produce semen having greater than 80% morphologically normal spermatozoa with satisfactory motility, live spermatozoa and concentration. The age, body weight and scrotal circumference (SC) of bucks were 15 to 28 months, 19.0 to 25.0 kg and 17.0 to 22.0 cm respectively. The bucks were reared in individual pen (4x2.5 sq.ft) and they were fed with Napier, Ipil-Ipil and/or German grass twice daily as per requirement. The feed was supplemented with commercial concentrate in pellet form (crude protein content: 120g/kg DM and energy content: 10.4 MJ ME/kg DM) in the morning and again in the afternoon at the rate of 100 gm/ buck. They were allowed for grazing and exercise for 1 to 2 hours daily. The breeding bucks were also supplied with germinated gram (20gm/buck/day) and clean and safe water.

### Preparation of diluters

Egg yolk-citrate diluter was used for the extension of semen. Before use, the egg yolk-citrate diluter was prepared according to Herman and Madden (1963). A stock solution for tris-glucose egg yolk diluents was prepared by dissolving tris-glucose and citrate in 85 ml distilled water.

### Semen collection and evaluation

Collection of semen was done with artificial vagina maintaining optimum temperature about 41-43°C. Semen was collected twice a week within 8.30 AM from each buck after cleaning the prepuce with antiseptic solution (Savlon). The individual ejaculate was evaluated immediately using the method by Herman and Madden (1963). One part of the splitted semen sample was diluted with egg yolk-citrate and another part with Tris diluter in a way so that 100 million motile spermatozoa per insemination dose were prevailed. Then the diluted semen was evaluated for motility (%), normal and abnormal sperm count (%), live and dead spermatozoa.

### Frozen semen production

For the production of frozen semen, semen samples were diluted with egg-yolk-citrate and tris diluter.

### Semen processing for frozen semen production

#### Dilution and preparation of semen

After evaluation, the semen was diluted on the basis of sperm concentration per ejaculate with the egg yolk-citrate, Tris and Triladyl based cryodiluent (diluter + cryoprotectant) to obtain a final concentration of 100 million spermatozoa per insemination dose. The motility (%) of the diluted semen was observed and recorded.

#### Filling and sealing

Extended semen was then filled manually into 0.50-ml straws and the laboratory ends of the straws were sealed with Polyvinyl chloride alcohol complexes (PVC) powder.

#### Equilibration

After sealing and filling, the straws were placed in the refrigerator at 4-5°C for 3 hours for equilibration. The motility of the equilibrated sperm was checked and only samples with more than 60-70% motility were used for freezing. After cooling the diluted sperm was placed in controlled rate freezer and initiated freezing.

#### Freezing

Equilibrated and diluted semen in plastic straws were loaded with the sealed end up in to the cryochamber, computer-controlled freezer (CL 3300) (plate-1) and cryogenesis version 5, to conduct the freezing trials. The straws were cooled from 5<sup>o</sup> to -80<sup>o</sup>C by cooling at 5<sup>o</sup>C per minute. After reaching -80<sup>o</sup>C, samples were removed from the cryochamber (plate 2); the frozen straws were immersed in liquid nitrogen (LN), followed by careful placing of the straws in to goblet to transfer in to the liquid nitrogen storage container.

#### Thawing

Straws were retrieved from the LN container using forceps after 24 hours and thawed in water bath at 37°C for 12 seconds. The straws were wiped and cut near the cotton plugged end. One drop of thawed semen was placed on to a previously warmed (37°C) slide and covered with cover slip. The post-thaw motility was estimated under microscope.

#### Insemination of does

After detecting estrus, the does were inseminated based upon behavioral signs and were observed at the AI center. During AI, the vulvar region of estrus, doe was cleaned with tissue paper. The doe was restrained by holding her hind legs upward. After that with the aid of a tubular speculum lubricated with non-spermicidal gel and equipped with a frontal light source, the external opening of cervix was visualized. In case of frozen semen, straw of frozen-thawed semen was loaded in AI gun covered with plastic sheath and placed carefully into os of the cervical canal. Double insemination was performed. After complete removal of semen, AI gun was removed from the vagina. A total of 96 does were inseminated with fresh diluted semen with EYC whereas 106 does were inseminated with frozen semen treated with tris diluter.

#### Conception rate measurement

Conception rate was recorded as the percentage of does that had not returned to estrus within 42 days (two cycles) after AI. This conception rate was also ensured by taking the history from the owner. Then conception rate was calculated by using the following formula:

$$\text{Conception rate (CR)} = \frac{\text{Number of does conceived}}{\text{Number of does inseminate d}} \times 100$$

### Statistical analysis

The data generated from this experiment were entered in Microsoft Excel worksheet, organized and processed for further analysis. Analysis was performed with the help of Statistical Analysis System Computer Package (SAS, 1998).

## RESULTS AND DISCUSSION

The semen characteristics for frozen semen of the experimental bucks are presented in Table 1.

### Evaluation of semen diluted with EYC diluter before freezing

After evaluation of fresh semen on the basis of different parameters like volume of semen, individual motility (progressive), sperm concentration and morphologically normal spermatozoa, one part of the splitted semen sample was diluted with the EYC diluter. Sodium citrate can disperse the fat globules in the egg yolk and make observation of individual sperm possible on microscopic examinations (Herman *et al.*, 1994).

**Table 1.** Evaluation of semen diluted with EYC diluter

Buck no	Diluted semen motility (%) (Mean±SD) (n=30)	Motility after cooling (%)	
		Without glycerol	With glycerol
3	70.00±0.00 <sup>b</sup>	67.00±2.00	63.75±7.75
4	72.50±2.45 <sup>ab</sup>	70.20±2.10	70.00±3.10
11	72.00±2.00 <sup>b</sup>	67.10±0.56	68.75±2.00
30	75.00±0.00 <sup>a</sup>	71.10±0.35	70.75±2.00
32	73.75±2.45 <sup>a</sup>	70.20±2.13	70.70±3.16
39	72.50±2.45 <sup>ab</sup>	70.00±2.00	70.00±3.16

Means with different superscripts within the same column differ significantly ( $p < 0.05$ )

SD = Standard deviation; n = Number of observations

With regard to the diluted semen, individual bucks showed significant difference ( $p < 0.01$ ) on volume and normal spermatozoa percentage and significant ( $p < 0.05$ ) difference was found on diluted semen motility with EYC diluter. Table 1 shows that the highest percentage of diluted semen motility was observed in buck 30 (75.00±0.00) and the lowest in buck 3 (70.00±0.00) with EYC diluter. On the other hand, the highest motility percentage without glycerol was reported in buck 30 (71.10±0.35) and the lowest in buck 3 (67.00±2.00). Similarly, the highest motility percentage with glycerol was obtained in buck 30 (70.75±2.00) and the lowest in buck 3 (63.75±7.75). This result is in agreement with the studies of pervious works (Das *et al.*, 2006, Afroz, 2005 and Banu *et al.*, 1988).

**Table 2.** Evaluation of semen diluted with Tris diluter

Buck no	Diluted semen motility (%) (n=30)	Motility after cooling	
		Without glycerol (%)	With glycerol (%)
3	73.50±1.25	69.80±2.00	67.70±2.20
4	74.00±1.50	71.00±1.90	70.00±3.15
11	74.25±2.00	72.00±1.80	70.50±2.20
30	75.75±2.20	74.00±2.20	72.25±2.20
32	73.80±2.20	72.50±2.00	70.50±2.00
39	74.50±2.20	72.00±2.20	70.00±3.15

SD = Standard Deviation; n = Number of observations

### Evaluation of semen diluted with Tris diluter before freezing

Present study showed that the highest diluted semen motility percentages was in buck 30 (75.75±2.20) whereas the lowest was in buck 3 (73.50±1.25) (Table 2). And the motility percentage without glycerol was the highest in buck 30 (74.00±2.20) and the lowest was in buck 3 (69.80±2.00). Whereas the motility percentages with glycerolated portion was the highest in buck 30 (72.25±2.20) and the lowest was in buck 3 (67.70±2.20) (Table 3). This study indicates that it is almost similar to Singh et al., (1996) and Afroz (2005).

### Evaluation of frozen semen

Means along with standard deviation (SD) of semen motility diluted with Tris and EYC diluter, motility after freezing, motility after thawing and live and normal sperm percentages are furnished in Table 3.

### Semen motility diluted with EYC and Tris diluter

Different experiments in this regard were performed and obtained better sperm viability with this diluter (Dutta et al., 1996, Singh et al., 1996 Afroz, 2005 and Janett et al., 2005).

**Table 3.** Comparative Evaluation of Frozen buck semen characters (using Tris and EYC diluter)

Diluter	Buck no	Motility after equilibration (%) (mean±SD)(n=30)	Motility after thawing (%)	Morphology	
				Live sperm (%)	Normal sperm (%)
EYC	3	57.50±8.00	37.50±4.90	29.65±2.49	75.89±2.59
	4	66.25±2.45	43.75±3.74	35.41±8.66	77.07±2.27
	11	63.75±2.00	42.50±5.83	33.33±10.98	78.53±1.45
	30	67.50±2.45	50.00±5.49	41.75±4.33	82.96±2.20
	32	67.40±2.45	47.50±5.10	41.75±3.40	79.48±0.92
	39	66.25±3.16	46.25±2.45	31.50±2.44	76.70±3.87
TRIS	3	66.50±2.00 <sup>a</sup>	48.75±5.48 <sup>b</sup>	40.19±2.46	74.65±2.68
	4	68.75±2.00 <sup>a</sup>	57.50±2.45 <sup>a</sup>	42.52±3.10	75.22±2.33
	11	69.00±0.00 <sup>a</sup>	56.25±2.45 <sup>a</sup>	41.11±2.13	77.53±2.51
	30	70.50±3.74 <sup>b</sup>	56.25±2.00 <sup>a</sup>	48.00±6.27	84.32±1.43
	32	70.00±0.00 <sup>a</sup>	57.50±0.46 <sup>a</sup>	42.44±3.84	82.06±0.85
	39	69.00 ±0.00 <sup>a</sup>	57.50±2.45 <sup>a</sup>	42.12±6.59	77.56±0.18

Means with different superscripts within the same column differ significantly ( $p < 0.05$ )

SD= Standard Deviation; n = Number of observations

### Motility after equilibration

The highest sperm motility after equilibration using egg citrate yolk diluter was obtained in buck 30 and the lowest in buck 3. The analysis of variance showed that the sperm motility after equilibration with EYC did not differ significantly among the bucks ( $p > 0.05$ ) (Table 3). This is in agreement with the findings of other investigators (Singh et al., 1996; Dutta et al., 1996, Biswas, 2001; Arriola and Foote, 1987 and Ahmad and Foote, 1985). In case of Tris diluter, the highest sperm motility after equilibration was obtained in buck 30 whereas the lowest in buck 3. It also supports the findings of Singh *et al.*, 1996; Dutta *et al.*, 1996 and Afroz, 2005. The analysis of variance showed that the sperm motility after equilibration with Tris differ significantly ( $p < 0.05$ ) among the bucks (Table 3). The difference could be due to the difference in the type of diluter, percentage of egg yolk, glycerol, equilibration time and thawing.

**Motility after thawing**

The analysis of variance revealed that the differences of sperm motility after thawing with EYC among the bucks were found insignificant ( $p>0.05$ ) (Table 3) whereas it was found to be significant ( $p<0.05$ ) with Tris diluter. This observation agrees with the result of Singh et al., (1996); Dutta et al., (1996) who found  $58.33\pm 1.67$  to  $60.00\pm 2.89\%$  sperm motility after thawing.

**Live sperm percentage (%)**

In this study, in case of Tris diluter, the highest and lowest live sperm percentages was in buck 30 ( $48.00\pm 6.27$ ) and 3 ( $40.19\pm 2.46\%$ ), respectively and in EYC it was as  $41.75\pm 3.40$  and  $29.65\pm 2.49\%$  in buck 30 and 3, respectively. This result agrees with Singh et al., (1996); Dutta et al., (1996).

**Normal sperm percentages**

This study showed that the highest and lowest normal sperm percentage using Tris diluter was  $84.32\pm 1.43$  and  $74.65\pm 2.68\%$  in buck 30 and 3 orderly whereas in case of egg yolk citrate diluter it was  $82.96\pm 2.20$  and  $75.40\pm 0.92\%$  in buck 30 and 3 respectively. This supports the finding of Singh et al., 1996; Dutta et al., 1996.

**Table 4.** Comparison of semen characteristics between EYC and Tris extender for frozen semen production

Characters	Extender		Level of significance
	EYC	Tris	
Motility after equilibration	$64.78 \pm 3.81$	$68.96 \pm 1.38$	*
Motility after thawing	$44.58 \pm 4.38$	$55.63 \pm 3.42$	**
Live sperm (%)	$35.57 \pm 5.16$	$42.73 \pm 2.73$	*
Normal sperm (%)	$78.44 \pm 2.57$	$78.56 \pm 3.85$	NS

P\*\* <0.01, P\* <0.05, NS = Non-significant

It revealed from statistical analysis that the motility after equilibration and live sperm percentages differed significantly ( $p<0.05$ ) when EYC and Tris diluter were considered for frozen semen production (Table 4). There was also significant variation ( $p<0.01$ ) between EYC and Tris on motility after thawing. No significant variation was found on normal sperm percentages.

**Productivity/Conception rate**

For the assessment of productivity, frozen semen using Tris and EYC diluter were used in this study. The spermatozoa concentration of frozen semen was (100 million/insemination dose). A total of 96 does were inseminated diluted semen with EYC whereas 111 does were inseminated frozen semen with Tris to determine the productivity.

**Table 5.** Conception rate in Black Bengal goat using semen diluted with EYC diluter

Buck No.	No. of does inseminated	No. of does conceived	Conception rate (%)
3	5	2	40.00
4	9	4	44.44
11	13	6	46.15
30	35	20	57.14
32	17	7	41.17
39	17	7	41.17
<b>Pooled</b>	<b>96</b>	<b>42</b>	<b>43.75</b>
<b>Level of significance</b>			<b>NS</b>

NS= Non-significant

### Productivity using egg yolk citrate diluter

Analysis of variance showed that the conception rate did not differ significantly ( $p>0.05$ ) by using frozen semen with EYC of 6 adult Black Bengal bucks. The highest conception rate was obtained by using semen of buck 30 (57.14%), followed by buck 11 (46.15%), buck 4 (44.44%), buck 32 (41.17%), buck 39 (41.17%) and buck 3 (40.00%) (Table 5). This variation in conception rate might be due to age, number of inseminations, quality of semen, and site of semen deposition and proper timing of insemination. Maxwell et al. (1996) had shown a linear relationship between conception rate and time of insemination.

### Conception rate using Tris diluter

The conception rate using frozen semen treated with Tris diluter is presented in Table-6 and variation of conception rate by using semen of the bucks were found to be insignificant ( $p>0.05$ ). In the present study, comparatively higher conception rate was obtained using frozen semen with Tris in buck 30 (61.52 %), followed by buck 11 (58.33%), buck 39 (54.17%) buck 32 (53.33 %), buck 4 (50.00%) and buck (40.00%) respectively (Table 6). So, the average conception rate of the Black Bengal goat using frozen semen made with Tris diluter was found 55.90% in the present study. This observation supports with the result of Khalifa and El-Saidy (2006), and Ritar et al. (1990). On the other hand, Dorado et al. (2007) and Sinha et al. (1987) obtained 42.9% and 42.1% conception rate using the frozen semen with Tris and EYC respectively which closely agrees with this study. Furthermore, the present result also collaborates with the result of other investigators (Singh et al., 1996 and Al-Faruque, 2004). This variation might be due to age, number of spermatozoa per dose, semen quality, and technique of AI employed, site and depth of semen deposition, number of inseminations performed and proper timing of insemination. Fieri et al. (1991) and Vallet et al. (1992) reported higher conception rate in intrauterine than cervical inseminations.

**Table 6.** Conception rate in Black Bengal goat using semen diluted with Tris diluter

Buck No.	No. of does inseminated	No. of does conceived	Conception rate (%)
3	5	2	40.00
4	16	8	50.00
11	12	7	58.33
30	39	24	61.52
32	15	8	53.33
39	24	13	54.17
<b>pooled</b>	<b>111</b>	<b>62</b>	<b>55.90</b>
<b>Level of significance</b>			<b>NS</b>

NS= Non-significant

**Table 7.** Comparison of pooled of two different extender for frozen semen production

Kind of semen treated with	No. of does inseminated	No. of does conceived	Conception rate
EYC	94	42	43.75 $\pm$ 6.37 <sup>NS</sup>
Tris	111	62	55.90 $\pm$ 7.49 <sup>NS</sup>

NS=Non-significant

The comparative result of the present study revealed that does inseminated semen diluted with EYC diluter conceived (43.75%) at a significantly ( $p < 0.01$ ) lower rate than those inseminated with frozen semen (55.9%) with tris diluter (Table 6). This is in agreement with the studies of Salamon and Maxwell (1995a) and Karatzas et al. (1997). This might be due to use of different concentration of spermatozoa per insemination dose and also of different diluters in these two kinds of semen. Tumen and Ozkoca (1994) correlated the conception rate with the concentration of spermatozoa in an AI dose. They reported higher concentration of spermatozoa in an AI dose tends to higher conception rate.

The promising observation from this study is also revealed that the fertilizing capacity of the sperm in two kinds of frozen semen was not same with non-significant ( $p > 0.05$ ) difference though the individuality or genetic potentiality of the same buck was higher in both kinds of semen. Among six Black Bengal bucks, buck 30 was screened as the best performer whereas 32, 39, 4, 11 was moderate and buck 3 was the poor performer. In this study, there were some limitations leading to different diluters and concentration of spermatozoa per insemination dose. The conception rate found both in Tris and EYC diluter is almost similar to abundantly used Triladyl diluter (58.25). Finally, it is possible to conclude that Tris diluter might be used as the alternative to the Triladyl diluter though further economic study to be needed for more confirmation.

## CONCLUSION

In case of fresh diluted semen with EYC diluter, individual bucks had significant effect ( $p < 0.05$ ) on diluted semen motility and but insignificant on motility after cooling with and without glycerol. On the other hand, it was insignificant ( $p > 0.05$ ) on diluted semen motility and motility after cooling with and without glycerol with Tris diluter. It revealed from statistical analysis that the motility after equilibration and live sperm percentages differed significantly ( $p < 0.05$ ) when EYC and Tris diluter were considered for frozen semen production. There was also significant variation ( $p < 0.01$ ) between EYC and Tris on motility after thawing. No significant variation was found on normal sperm percentages. Significant variations of semen parameters were observed between diluter to diluter and buck to buck. No significant variation was found on normal sperm percentages. The efficacy of tris extender is better than that of EYC diluter taken into considerations all semen attributes. The productivity with EYC was 43.75% but with frozen semen made with Tris was 55.90%. When the conception rate of semen diluted with EYC and Tris was considered separately, no significant difference ( $p > 0.05$ ) of conception rate by the bucks was observed. But when the conception rate was compared between the two kinds of frozen semen (diluted with EYC and Tris) it was revealed that the kind of semen had no significant effect ( $p > 0.05$ ) on conception rate. Considering the conception rate, it was reported that the highest conception rate was obtained by the semen of buck 30 and lowest by buck 3, though further study is to be needed for more economic confirmation.

## CONFLICT OF INTEREST

The authors have no affiliations with or involvement in any organizations or entity with any financial and non-financial interest in the subject matter or materials stated in this manuscript.

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