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Detection and Quantification of X and Y sperms in Enriched Sahiwal Semen diluted in PBS using Raman Spectroscopy

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ABSTRACT

The present study was undertaken to enrich X-sperms in Sahiwal bull semen by percoll density gradient method and to detect X and Y sperms in enriched semen by Raman spectroscopy. The experiment was performed at Semen Production Centre, CVASc, GBPUAT, Pantnagar, Uttarakhand. Ejaculates were collected by AV method from Sahiwal bull. X-sperm enrichment was done by the Percoll density gradient method. Three types of gradients were prepared i.e. Group I: Seven layers (70-10%), Group II: Seven layers (80-20%) and Group III: Three layers (30%, 50% and 70%). Semen (1ml or 3ml) was placed over the gradient in a conical centrifuge tube. Centrifugation was done at 750 g (22-24°C) for 15 min. The pellets thus obtained were diluted in PBS medium. Raman spectroscopy was performed in different groups viz. fresh semen (Control), and three treatment groups. Raman peaks (DNA specific) were not much clear for PBS diluted semen samples though intensities were highest for seven layers (70-10%) among all groups. Thus, semen enriched by the percoll-density gradient method (7 layers i.e. 70-10%) can be used to increase female calf birth after A.I.

Key words: Raman spectroscopy, Sahiwal, Semen enrichment, X Y Sperm.

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INTRODUCTION

India is the highest milk producer and ranks first position in the world contributing 24% of global milk production in the year 2021-22. The milk production of India has registered 61% increase during the last eight years i.e., during the year 2013-14 and 2021-22 and increased to 221.1 Mn Tonnes in the year 2021-22. In order to meet it the global food demand associated with this population growth, it will be necessary to increase the number of elite

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female by shifting the sex ratio towards females. Gender selection is one of the biggest outcomes of research in livestock sector during the last two decades and came to the stage of broad commercial application. Semen having sperms of a desired sex (with 80-90% accuracy) is known as sexed semen which predetermines the sex of the offspring prior to conception.

In the livestock industry, aim of predetermination of sex of foetus has been a main goal of dairy sector and livestock owners for generations to produce a calf of specific sex.

Production of unwanted male calves should be minimized because they cannot be slaughtered in India. Production of breeding bulls can be conserved (Singh *et al*., 2017) as in India the number of elite cattle and buffaloes are limited (Boro *et al.,* 2016, Sharma *et al*. 2018; Chaudhary *et al*., 2022).In the dairy sector, selection of desired calf and reduced male calves results in high productivity per unit area (Rath *et al.,* 2009).

In India, land productivity is decreasing. Thus fodder and other feed resources for the animals are restricted. Therefore, it is difficult to fulfill the feed requirement of both unproductive bulls and elite animals (Singh *et al*., 2017). Currently, the use of flow cytometry to separate X- and Y-sperm is the only successful method of sex pre-selection with accuracy of 90% but because of some limitations it is difficult to commercialize. In flow cytometry, motility of sperms gets highly decreased compared to non-sexed semen (Sharma and Sharma, 2016; Sharma *et al.,* 2018, Rawat and Sharma, 2020, Bhat and Sharma, 2020). Hence present method of X sperm enrichment is more beneficial in terms of progressive motility. So, there is an urgent need of technological intervention for reduction of unwanted bulls.

The resultant 'sex-sorted' spermatozoa are then able to be used in conjunction with other assisted reproductive technologies such as artificial insemination or in-vitro fertilization (IVF) to produce offspring of the desired sex (Sharma *et.al*., 2022). Hence the present study was undertaken to enrich X-sperms in Sahiwal bull semen by percoll density gradient method and to detect and quantify X and Y sperms in enriched semen by Raman spectroscopy in Sahiwal bull semen.

MATERIAL AND METHODS

The present study was performed at Semen Production Center, Department of Veterinary Gynaecology & Obstetrics, CVASc (College of Veterinary and Animal Sciences), G. B. Pant University of Agriculture and Technology, Pantnagar, Uttarakhand and UGC-DAE Consortium, Indore (M.P.).

Collection of Semen: Semen was collected thrice a week during morning hours from healthy a Sahiwal bull and 6 numbers of samples for each group had been utilized for the study. The ejaculates having the mass activity of >+3 and a progressive motility of >70% were considered for further processing (number of bulls and number of samples utilized for the study.

Separation of Two Populations of Semen: The collected ejaculates were subjected for three gradient groups and control.

Group I (3 gradient layers): Three ml of each gradient i.e., 70, 50 and 30% percoll solution were taken in a 15 ml conical centrifugation tube with densest at bottom (70%) and lighter at top (30%). Semen (3 ml) was layered on the top of conical centrifugation tube containing percoll gradient. Then centrifugation of this conical centrifugation tube containing semen and percoll gradient was done for 15 minutes at 750 x g (2955 RPM) at 22-24°C.

Group II (7 gradient layers): One ml of each gradient i.e., 70, 60, 50, 40, 30, 20 and 10% percoll solution were taken in a 15 ml conical centrifugation tube with densest at bottom (70%) and lighter at top (10%). Semen (1 ml) was layered on the top of conical centrifugation tube containing percoll gradient and subjected for centrifugation as detailed previously.

Group III (7 gradient layers): One ml of each gradient i.e., 80, 70, 60, 50, 40, 30 and 20% percoll solution were taken in a 15 ml conical centrifugation tube with densest at bottom (80%) and lighter at top (20%). Semen (1 ml) was layered on the top of conical centrifugation tube containing percoll gradient and subjected for centrifugation as detailed previously (Lizuka *et al.,* 1987 and Bhat and Sharma, 2020).

Semen samples were collected after centrifugation using a pipette or a syringe fitted with a Wide-bore needle. It is important to keep the Tip of the instrument against the wall of the Tube just above the surface of the liquid to avoid at above the surface of the interface.

Detection and Quantification of X and Y sperm in Enriched Semen by Raman Spectroscopy: In each group, samples were prepared having equal number of sperms and volume i.e. $2x10^6$ sperms were mixed in 400 µl of phosphate buffered saline (PBS). These were prepared after determining the sperm concentration. Samples prepared one week advanced were stored at -20°C in PBS.

All the samples were brought to the UGC-DAE Consortium, Indore (M.P.) to perform Raman Spectroscopy. Four µl of semen was loaded in chamber to get Raman Spectra. It was performed at 25 watt voltage,

473 nm laser with 30 seconds exposure time and 100X objective lens with of 0.9 numerical aperture of confocal Microscope. Spectra was taken by using wave number from 500-1800 cm^{-1} (De Luca *et al.,* 2014).

RESULTS AND DISCUSSION

The Raman analysis is a useful diagnostic method for determining the structure and composition of biomolecules. A variety of studies have been conducted to demonstrate the viability and high reliability of biological cell characterization. In the present paper, we have analyzed the Raman spectroscopic characteristics of Sahiwal semen. Raman spectroscopy of samples was performed in four groups to investigate the effect of percoll density gradient method on X sperm enhancement in Sahiwal semen: PBS diluted pellet of fresh semen (control), 3 layer 70 percent percoll pellet (Group I), 7 layer 70 percent percoll pellet (Group II).

Figure 1 shows Raman spectrum of control Sahiwal sperm cells. The prominent Raman peaks located at around 602, 652, 679, 726, 782, 981, 1095, 1331, 1360, 1450, 1581, and 1650–1770 cm^{-1} are observed in control Sahiwal sperm cells, with the strongest signals at 782, 1095 1360 and 1650–1770 cm^{-1}. Raman peak assignment for all the wave number is shown in the Table 1. Raman peak at 782 cm^{-1} was attributed to thymine, cytosine, as well as the DNA backbone [15], 1095 cm^{-1} was indicative of the PO_2^- backbone, 1360 cm^{-1} was generally assigned to Thymine, Adenine, Guanine and a broad band 1650-1770 assigned for Thymine, Adenine, Guanine, Cytosine.

Table 1: Raman Band assignment in PBS diluted pellet of fresh semen of Sahiwal (control).

S. No.	Wave number (cm^{-1})	Raman peak assignment
1	602	Cytosine
2	652	Thymine
3	679	Guanine
4	726	Adenine
6	782	Thymine, Cytosine, DNA backbone
7	895	Phenylalanine
8	981	CO
9	1095	PO_2^- backbone, Proteins, =CH
10	1331	Adenine, CH
11	1360	Thymine, Adenine, Guanine
12	1450	Protein
13	1581	Adenine, Guanine
12	1650-1770	Thymine, Adenine, Guanine, Cytosine, CH, CH_2

This Raman band in the middle has a high peak amplitude. The Raman spectrum for Group I, Group II, and Group III is shown in Figure 2. The Raman spectrum of different groups was analyzed, and it was discovered that the Raman bands are nearly identical in all of them. The Raman strength, on the other hand, varied across all categories. Table 2 and Figure 3 show the comparison of intensities at DNA unique peaks. Intensities at DNA specific peak were compared. In all of the classes, there was a substantial difference in intensity at DNA specific wave numbers. At wave number 782 cm^{-1}, the values for influence, control, group I, group II, and group III were 49, 88, 46 and 80, respectively. At 1095, the intensities were 442, 611, 631 and 548, respectively, at 1360, the intensities were 162, 179, 110 and 143 respectively while at 1650-1770, the intensities were 90, 215, 150 and 200, respectively, in control, group I, group II, and group III. Raman peaks in enriched PBS diluted semen were not much clear to quantify the enrichment but Raman spectrum, showed that intensities for corresponding wave number were highest for the enrichment by the 7 layers (70%) followed by 3 layers (70%), 7 layers (80%) and control.

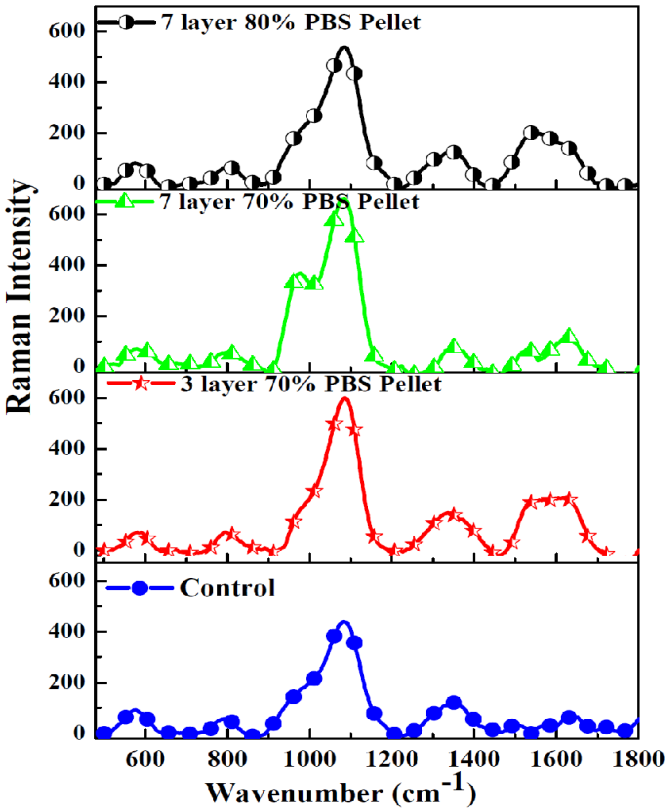

Fig 1: Raman Spectra of X-sperm enriched PBS diluted Sahiwal semen.

Quantification of enrichment in Sahiwal Species in PBS medium

Table 2: Effect of X sperm enrichment by percoll density gradient method on Raman peaks of PBS diluted Sahiwal semen at DNA specific wave numbers.

Wave number (cm^{-1})	Control	Group I	Group II	Group III
782	49	88	46	80
1095	442	611	631	548
1360	162	179	110	143
1650-1770	90	215	150	200

Table 3: Area under the curve for different Raman bands in control, Group I, Group II and Group III at some specific wave numbers.

Wave number (cm^{-1})	Control	Group I	Group II	Group III
782	3369.21	4620.66 (37.14%)	4110.66 (22.00%)	4340.10 (28.81%)
1095	59718.26	66703.47 (11.69%)	88274.39 (47.01%)	71919.98 (20.43%)
1360	12756.37	16216.18 (27.52%)	3050.25 (76.09%)	13016.78 (2.04%)
1650-1770	11685.44	21889.78 (87.32%)	13055.13 (11.72%)	22912.67 (96.07%)

To confirm the enhancement of the X sperms in Sahiwal semen, we additionally calculated the area under the peaks at approximately 782, 1095, 1360 and 1650-1770 cm^{-1}, which are clearly shown in the Table 3. Normally, the number of collisions of a single bond is represented by the quantification area under the curve for signature peak. The area under the peaks differs between X-bearing and Y-bearing sperm, indicating the relative difference in DNA content. (De Luca *et al.,* 2014). Origin Pro 2008 was used to determine the area under the curve. Table 3 shows the region under the curve values for specific peaks of control, 3 layer 70% percoll pellet, 7 layer 70% percoll pellet and 7 layer 80% percoll pellet following the calculation of the area under the curve, the percentage area variation by GI, GII, GIII with the control was determined using the formula given below:

$$\% \text{ Area variation} = \frac{[(\text{Area under the curve in GI, GII, GIII})_{\text{particular frequency}} - (\text{Area under the curve for Control})_{\text{particular frequency}}]}{(\text{Area under the curve for Control})_{\text{particular frequency}}}$$

Parenthesis of the Table 3 indicates the percent area difference for each frequency. The % area variation is high for the case of 3 layer 70% percoll pellet and 7 layer 70% percoll pellet than 7 layer 80%. Peak intensity and percent area variation were higher in X-bearing sperm cells than in Y-bearing sperm cells, which was due to higher DNA concentration in X-bearing sperm cells. As a result, the Raman procedure can be used to classify X-bearing bovine sperm cells.

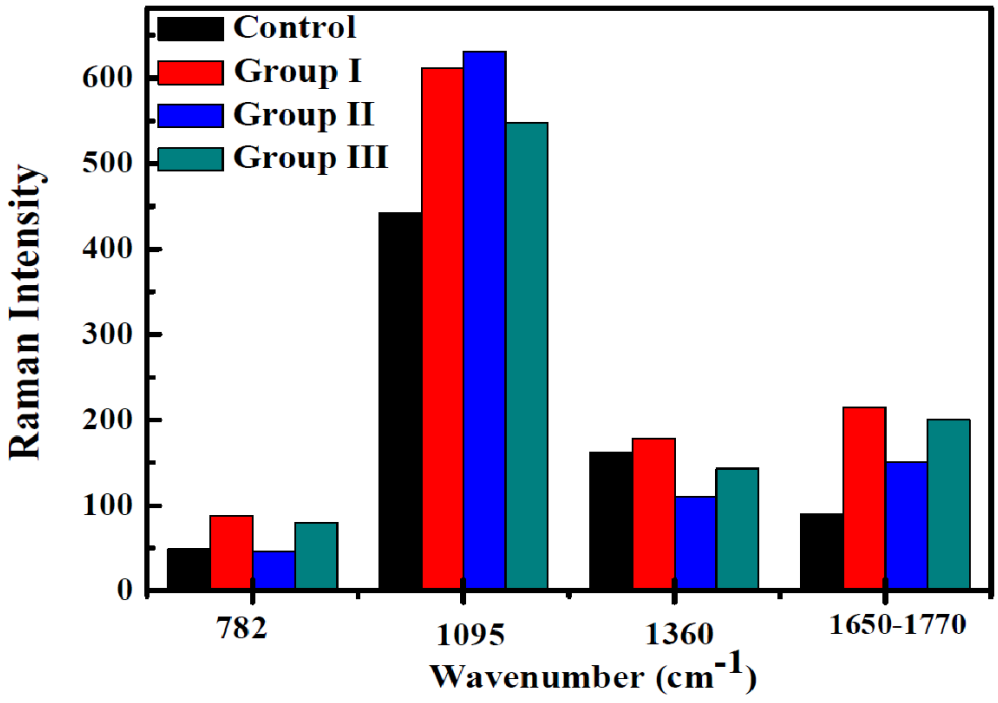

Fig 2: Effect of X-sperm enrichment by percoll density gradient method on Raman peaks of PBS diluted semen at DNA specific wave number.

CONCLUSIONS

Despite the Raman peaks (DNA specific) were not much clear for PBS diluted Sahiwal semen sample, intensities were highest for 7 layers (70-10%) among all groups. An implication of this is the possibility that semen enriched by the percoll density gradient method (7 layers i.e. 70-10%) can be used to increase the birth rate of female calves following artificial insemination. Moreover, the authors recommend that further studies need to be carried out in order to validate the findings of the present study.

CONFLICT OF INTEREST

The authors declare no conflict of interest in the conduct of this experiment.

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