RESEARCH ARTICLE Indian Journal of Animal Research, Volume 55 Issue 12: 1401-1408 (December 2021)

# Evaluation of Level of Pesticide Residue in Seminal Fluid of Crossbred Bulls

Paramveer Singh Sangha, Ajeet Kumar, Mrigank Honparkhe, Jasbir Singh Bedi[1], Ashwani Kumar, Prahlad Singh

10.18805/IJAR.B-4518

## ABSTRACT

**Background:** Pesticides act as endocrine disruptors and cause enormous disturbances in steroidogenesis, spermatogenesis and sexual behavioural display. These toxicants modulate and/or disrupt the reproductive and hormonal environment by acting on hypothalamus, pituitary and reproductive organs. Few studies have been carried out to assess the levels of pesticides in dairy cattle/ buffaloes. The information on pesticide residues in body fluids especially blood and semen in breeding bulls is lacking.

**Method:** In the light of above background nineteen fresh semen ejaculates were collected from Milkfed, Khanna and PLDB (Punjab Livestock Development Board), Nabha, Punjab and stored at -80°C. Immediately after collection, the semen was centrifuged at 4500 × g for 20 min at 4°C. The supernatant (seminal plasma) was stored at -20°C until processing for the assessment of pesticide residues using gas chromatography (GC). In the present study on crossbred breeding bulls, the semen samples were analyzed for organochlorines, organophosphates and synthetic pyrethroid pesticide residues. Seven OCP were screened namely Heptachlor epoxide, Chlordane, Fipronil, Lindane, Methoxychlor, op-DDT, Endrin. Eleven OPP were screened namely Chorpyrifos, Dichlorovos, Ethion, Monocrotophos, Malathion, Parathion-methyl, Profenphos, Phorate, Triazophos, Quinalphos and Phosalone and four SP's namely Cypermethrin, Permethrin, Deltamethrin, Cyalothrin were screened in the semen samples. Semen samples were also assessed for viability, HOST (%) and abnormality (%) for both fresh as well as post thaw samples.

**Result:** None of the semen samples were found positive for any of the above pesticide residues. This may be attributed to the feeding of fodder cultivated at the stations without using any pesticides, hence no pesticide residue found in the semen samples of the crossbred breeding bulls of these semen stations in present study, viability (%) of sperms were found to be significantly higher ($p<0.05$) in fresh (75.57±5.31) as compared to post thaw (70.17±4.64) semen of bulls. The abnormality (%) of sperms was found to be significantly lower ($p<0.05$) in fresh (13.61±3.71) than post thaw (18.22±3.59) semen bulls. Also in present study the host (%) was found 67.23±4.76 in fresh semen which decreased to 46.82±3.55 in post thaw semen.

**Key words:** Crossbred bulls, Gas chromatography, Pesticide residue.

## INTRODUCTION

Pesticides act as endocrine disruptors and cause huge disturbances in steroidogenesis, spermatogenesis and exhibition of sexual behavior. Due to inadvertent use of potentially dangerous pesticides, dairy animals are highly vulnerable to their exposure *via* soil, food, water and animal-derived oil or fat products incorporated into processed feeds (Ratnakaran *et al.* 2014). These pesticides include not only old and biologically persistent organochlorine pesticides like dichloro diphenyl trichloroethane (DDT), hexa chlorocyclo hexane (HCH), heptachlor, aldrin and endosulfan, but also highly toxic organophosphorus pesticides (OPP) like phosphamidon, chlorpyrifos (CPF) and malathion. These toxicants modulate and/or disrupt reproductive and hormonal milieu by acting at a variety of sites including hypothalamus, pituitary and reproductive organs (Zama and Uzumcu 2010). Only few studies have been conducted to assess the pesticide levels in blood of dairy cattle/buffaloes, however no information is available related to breeding bulls. In view of the above information, the present study was designed to evaluate the level of pesticide residues in seminal plasma of crossbred breeding bulls to assess the impact of pesticide levels on semen quality of crossbred breeding bulls.

Department of Veterinary Gynaecology and Obstetrics, Guru Angad Dev Veterinary and Animal Sciences University, Ludhiana-141 001, Punjab, India.

[1]School of Public Health and Zoonoses, Guru Angad Dev Veterinary and Animal Sciences University, Ludhiana-141 001, Punjab, India.

**Corresponding Author:** Paramveer Singh Sangha, Department of Veterinary Gynaecology and Obstetrics, Guru Angad Dev Veterinary and Animal Sciences University, Ludhiana-141 001, Punjab, India. Email: paramveersangha7@gmail.com

**How to cite this article:** Sangha, P.S., Kumar, A., Honparkhe, M., Bedi, J.S., Kumar, A. and Singh, P. (2021). Evaluation of Level of Pesticide Residue in Seminal Fluid of Crossbred Bulls. Indian Journal of Animal Research. 55(12): 1401-1408. DOI: 10.18805/IJAR.B-4518.

**Submitted:** 11-05-2021 **Accepted:** 17-07-2021 **Online:** 09-08-2021

## MATERIALS AND METHODS

### Experimental design

Nineteen fresh semen ejaculates were collected from Milkfed, Khanna and PLDB (Punjab Livestock Development Board), Nabha, Punjab and stored at -80°C. Immediately after collection, the semen was centrifuged at 4500 × g for 20 min at 4°C. The supernatant (seminal plasma) was stored

at -20°C until assay. Pesticide residues were evaluated using gas chromatography (GC) and Gas chromatography-mass spectrometry (GC-MS) (Kamarianos *et al.* 2003a) (Table 1) in the Pesticide Residue Testing Laboratory, School of Veterinary Public Health, Guru Angad Dev Veterinary and Animal Sciences University, Ludhiana, Punjab in 2019-2020.

**1. Estimation of pesticide residues in semen samples**

**Sample treatment**

Each sample was extracted with methanol and centrifuged after thawing at temperature and once homogenised. The extract was next passed through a solid phase extraction cartridge filled with 500 mg of C18 guaranteed porous silicon oxide, Analytichem Bond Elut LRC from Varian (Harbour city, CA, USA), to separate and concentrate the analytes, per the theme shown in Fig 1 (Guardino *et al.* 1996) with slight modifications. To carry out this step, the extracts were classified in batches of 10. Each and every batch was treated by suggests that of a vacuum process station (Analytichem vacation Elut SPS24, Varian) at a consistent flow-rate of 2 ml/min. The extracts were processed simultaneously, but the flow was controlled individually and, for each sample, the process was stopped by means of a valve when the extract came level with the surface of the solid phase inside the cartridge. The elutes were collected in pre-weighed vials so as to control the final volume, after concentration using nitrogen flow. Each sample batch was analyzed along with a blank of methanol following constant process.

**Estimation using gas chromatography**

Gas chromatography detection was used for the pesticide residues in all the analyzed commodities. Gas chromatography is a technique used for separating and analyzing compounds that can be vaporized without decomposition. The compound must have sufficient volatility and thermal stability. In gas chromatography, the mobile phase is a carrier gas, usually an inert gas such as helium or a non-reactive gas such as nitrogen. The stationary phase is a microscopic layer of liquid or polymer on an inert solid support, inside a piece of glass or metal tubing called a column. Fully programmed auto injector injects the sample using micro syringe through a rubber septum into a flash vaporizer port at the head of the column. Once in the column, the compounds in the mixture are separated by differences in the positions of adsorption, equilibrium between the gaseous components of the sample and the stationary phase. In present study, cleaned up extracts were analyzed by GC equipped with ECD for OCPs, SPs and FTD for OPs compounds with standardized operating conditions (Table 2).

**Sample introduction to GC**

The cleaned up extract measuring 1-2 µl was injected in GC similarly as standard solution through auto injector.

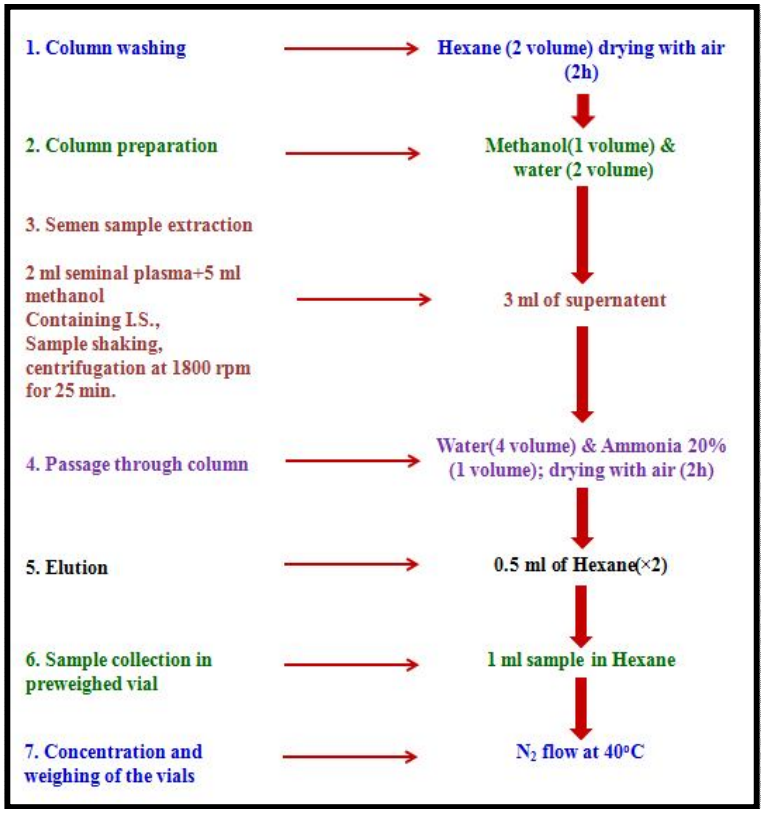


**Fig 1:** Sample treatment and sample clean up scheme for seminal plasma for estimation of pesticide residues (by modified method of Guardino *et al.,* 1996).

**Table 1:** List of pesticides standard used in present study.

| Organochlorine pesticides | Organophosphorus pesticides | Synthetic pyrethroids pesticides |
|---|---|---|
| Heptachlor epoxide | Chorpyrifos | Cyhalothrin |
| Chlordane | Dichlorovos | Permethrin |
| Fipronil | Ethion, | Cypermethrin |
| Lindane | Monocrotophos | Deltamethrin |
| Methoxychlor | Malathion | |
| op-DDT | Parathion-methyl | |
| Endrin | Profenphos | |
| | Phorate | |
| | Triazophos | |
| | Quinalphos | |
| | Phosalone | |

**Table 2:** Gas chromatography conditions for organochlorines, synthetic pyrethroids and organophosphorus pesticides detection.

| | | OCPs and SPs | | | OPs | | |
|---|---|---|---|---|---|---|---|
| Auto injector | Injection volume | 2 µl | | | 1 µl | | |
| | No. of rinses with solvent (pre run) | 5 | | | 5 | | |
| | No. of rinses with solvent (post run) | 10 | | | 10 | | |
| | No. of rinses with sample | 2 | | | 2 | | |
| | Plunger speed (suction and injection) | High | | | High | | |
| | Syringe insertion speed | High | | | High | | |
| | Injection mode | Normal | | | Normal | | |
| Injection port | Temperature | 280°C | | | 280°C | | |
| | Injection mode | Split | | | Split | | |
| | Carrier gas | $N_2$ | | | $N_2$ | | |
| | Flow control mode pressure | 117.4 kPa | | | 117.4 kPa | | |
| | Total Flow | 14.0 ml/m | | | 5.2 ml/m | | |
| | Column flow | 1.00 ml/m | | | 1.08 ml/m | | |
| | Linear velocity | 30.7 cm/sec | | | 31.7 cm/sec | | |
| | Purge flow | 3.0 ml/m | | | 3.0 ml/m | | |
| | Split ratio | 10.0 | | | 1.0 | | |
| Column | Type | RTX-5 | | | RTX-5 | | |
| | Length | 30.0 meter | | | 30.0 meter | | |
| | Inner diameter | 0.25 mm ID | | | 0.25 mm | | |
| | Film thickness | 0.25 µm | | | 0.25 µm | | |
| | Equilibration time | 0.5 m | | | 0.5 m | | |
| | Temperature (programmed) | Rate (temp/m) | Temp (°C) | Holdtime (m) | Rate (temp/m) | Temp (°C) | Holdtime (m) |
| | | | 170 | 13.0 | | 150 | 5.0 |
| | | 3.0 | 270 | 20.0 | 10.0 | 220 | 3.0 |
| | | | | | 5.0 | 240 | 13.0 |
| Detector | Type | ECD | | | FTD | | |
| | Temperature | 310°C | | | 300°C | | |
| | Sampling rate | 40 msec | | | 40 msec | | |
| | Current | 1.00 pA | | | 1.30 pA | | |
| Gas | Hydrogen | | | | 3.0 ml/m | | |
| | Air | | | | 145ml/m | | |

Residues were quantitatively determined by comparison of the retention time (Table 3) and peak areas/heights of the sample chromatogram with those of standard solutions run under the same operating conditions. The concentration of pesticide residues was reported as ng $g^{-1}$ on fresh weight basis. The formula for quantification of residues:

$$\text{Residues (ng/ml)} = \frac{\text{(Peak area of the sample)} \times \text{(ng of pesticide standard injected)} \times \text{(Final volume of extract, ml)}}{\text{(Peak area of the standard)} \times \text{(Volume of the sample injected)} \times \text{(Initial volume, ml)}}$$

**2. Post thaw semen quality (Post-thaw motility, viability and membrane integrity) in relation to pesticide residues of crossbred breeding bulls**

**Methodology**

Nineteen fresh semen ejaculates, from Milkfed, Khanna and PLDB (Punjab Livestock Development Board), Nabha, Punjab were collected and stored at -80°C. The post thaw semen quality was assessed in Department of Veterinary Gynaecology and Obstetrics, Guru Angad Dev Veterinary and Animal Sciences University, Ludhiana, Punjab.

Individual sperm motility and sperm viability were estimated in fresh ejaculates and after thawing using standard procedure and semen was subjected to Hypo-Osmotic Swelling Test (HOST) for plasma membrane integrity (PMI) and for HOST following procedure was used:

a. Two hypo-osmotic solutions were prepared as follows: 2.7% aqueous solution of fructose (1.351 gm/50 ml distilled water) and 1.47% aqueous solution of sodium citrate (0.735 gm/50 ml distilled water).
b. Equal volumes of both solutions (0.5 ml each) were mixed and kept in an incubator at 37°C for 10 minutes.
c. 50 µl of semen was added in above hypo-osmotic solution (Fructose + Sodium citrate) and incubated at 37°C for 30 minutes.
d. 10µl of this mixture was taken on glass slide and covered with glass cover.
e. Slide was observed under 10 X objective lens to determine the number of spermatozoa showing swollen head and coiled tail indicating sperms with intact plasma membrane (HOS positive sperm).
f. Total hundred spermatozoa were counted to determine the percentage of HOST positive spermatozoa.

## RESULTS AND DISCUSSION

**Pesticide residue in semen of crossbred breeding bulls**

In the present study on crossbred breeding bulls, the samples were analyzed for the pesticide residues. Seven OCP were screened namely heptachlor epoxide, chlordane, fipronil, lindane, methoxychlor, op-DDT, endrin. Eleven OPP were screened namely chorpyrifos, dichlorovos, ethion, monocrotophos, malathion, parathion-methyl, profenphos, phorate, triazophos, quinalphos and phosalone and four SP's namely cypermethrin, permethrin, deltamethrin, cyalothrin were screened in the semen samples and none of the semen samples were found positive for any of the above pesticide residues. This may be attributed to the feeding of fodder cultivated at the stations itself where pesticides were not used, therefore no pesticide residue found in the semen samples of the crossbred breeding bulls. Also, the use of biologically persistent OCPs (DDT and HCH) on crops is banned worldwide due to their potential for bioaccumulation, trans-boundary movement and biological effects (Thullner 1997). In this study, OPP used were not detectable in the semen samples of animals. This could be attributed to the blood-testis and epididymis barrier (Kamarianos *et al*. 2003a) and short biological half-life of OPPs (Smith 1995). The graphs for the sample analysis are presented (Fig 2-5).

**Evaluation of semen**

Semen evaluation of crossbred bulls is presented in Table 4. In present study, individual sperm motility (was found to be significantly higher ($p<0.05$) in fresh semen (90.21±4.31%) than the post thaw (57.89±5.23) semen. Similar observation was also reported by Kumar *et al*. (2008).

**Table 3:** Retention time of various pesticide standards in gas chromatography (GC) procedures.

| Pesticides | | | | | |
|---|---|---|---|---|---|
| Organochlorine pesticides | Retention time (min) | Organophosphorus pesticides | Retention time (min) | Synthetic pyrethroids pesticides | Retention time (min) |
| Heptachlor epoxide | 18.881 | Chorpyrifos | 7.048 | Cyhalothrin | 38.104 |
| Chlordane | 21.949 | Dichlorovos | 2.478 | Permethrin | 40.233 |
| Fipronil | 21.17 | Ethion, | 10.829 | Cypermethrin | 43.555 |
| Lindane | 8.168 | Monocrotophos | 5.047 | Deltamethrin | 48.666 |
| Methoxychlor | 33.763 | Malathion | 7.759 | | |
| op-DDT | 27.14 | Parathion-methyl | 8.092 | | |
| Endrin | 25.102 | Profenphos | 9.875 | | |
| | | Phorate | 5.241 | | |
| | | Triazophos | 11.129 | | |
| | | Quinalphos | 8.957 | | |
| | | Phosalone | 13.837 | | |

**Individual motility (%)**

In present study, individual sperm motility was found to be significantly higher ($p<0.05$) in fresh semen (90.21±4.31%) than the post thaw (57.89±5.23) semen. Similar observation was also reported by Kumar *et al.* (2008).

The results of the present study for initial motility are higher than those reported by Angasaria *et al.* (2002). They observed initial motility in the range of 30-83% with an average of 67.24±2.59 and 66.80±2.28, in HF crossbred bulls. In a previous study, the mean initial motility was recorded to be 67.60±0.47 (%) in HF crossbred bulls (Kanchan and Matharoo 2015). The results in the present study for initial motility is higher than Vyas *et al.* (1992) who reported 60.69±1.43 (%) initial motility in HF crossbred bulls. Shaha *et al.* (2008) found 56.6 to 76% initial motility in HF crossbred bulls. Hossain *et al.* (2012) observed 63.7% initial motility in 97 breeding bulls at the Central Cattle Breeding and Dairy farm, Savar, Dhaka. Another study reported 78.2, 75, 80 and 80 (%) initial motility in Pure HF, 50% cross, 75% cross and Borena bulls, respectively at NAIC, Kaliti, Addis Ababa, Ethiopia (Alemu *et al.* 2014). In another study, the initial motility was found to be 60.39±0 % in HF crossbred bulls (Chauhan *et al.* 2017). The variation in the initial motility may be due to variation between individual bulls, percent live sperms, method of detection (CASA and manual), season and technician.

The results of post thaw motility (%) in present study (57.89±5.23) are slightly higher than those reported by Mathur *et al.* (2014) who found the mean post-thaw motility in exotic breeds (HF and Jersey) was 52.40% followed by 50.85% percent in crossbreds (HF, Jersey and other crosses). The overall mean post-thaw motility was 51.02%. They reported that, among the semen production stations, the average post-thaw motility of frozen semen varied from 52.70 to 49.70%. In another study, it was reported that 53.8% of tested bulls exhibited >40% post thaw motility in 14 cross-bred and 12 pure bred bulls (Zodinsanga *et al.* 2015). Gopinathan *et al.* (2016) observed 49.8±0.0% post thaw motility in crossbred Holstein Friesian bulls. The variation in the post thaw motility might be due to variation between individual bulls, cryopreservation techniques, period of storage, thawing methods and maintenance of liquid nitrogen.

**Table 4:** Individual motility (%), Viability (%), Total sperm abnormalities (%) and Membrane integrity(%) of semen samples collected from crossbred breeding bulls(n=19).

| Parameters | Fresh | Post thaw |
|---|---|---|
| Individual motility (%) | 90.21±4.31 | 57.89±5.23* |
| Viability (%) | 75.57±5.31 | 70.17±4.64 |
| Total sperm abnormalities (%) | 13.61±3.71 | 18.22±3.59 |
| HOS% | 67.23±4.76 | 46.82±3.55* |

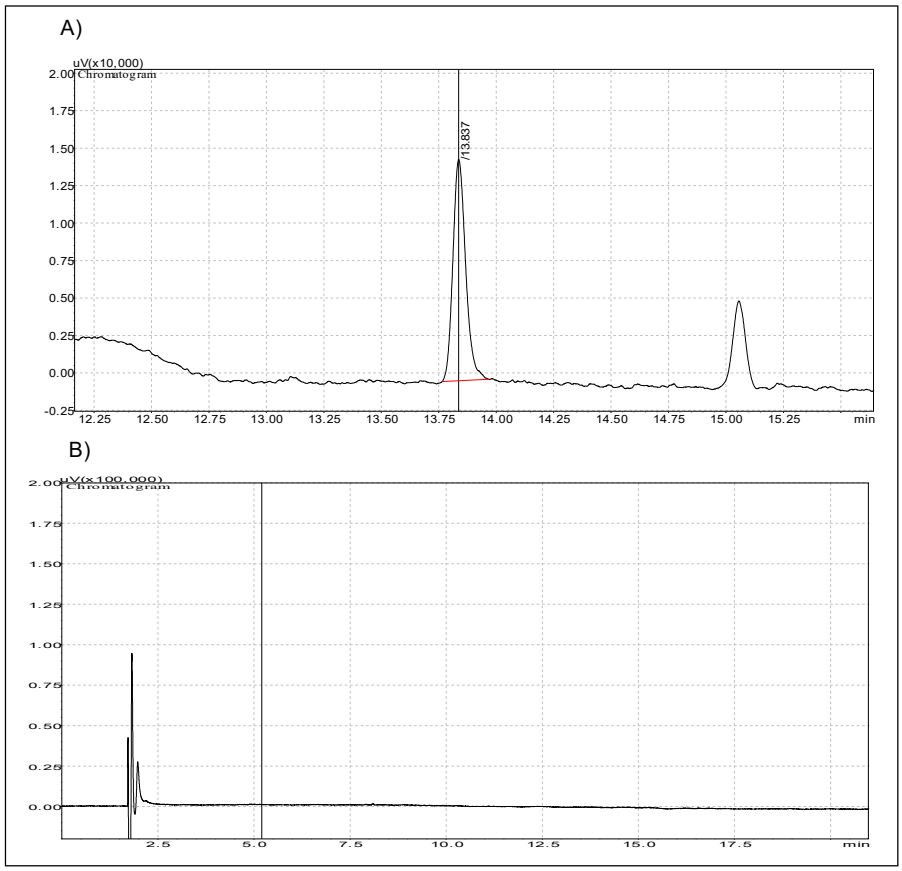


**Fig 2:** Chromatogram of A) Standard (at retention time of 13.83 min.) and B) Semen (no peak detected) for phosalone.

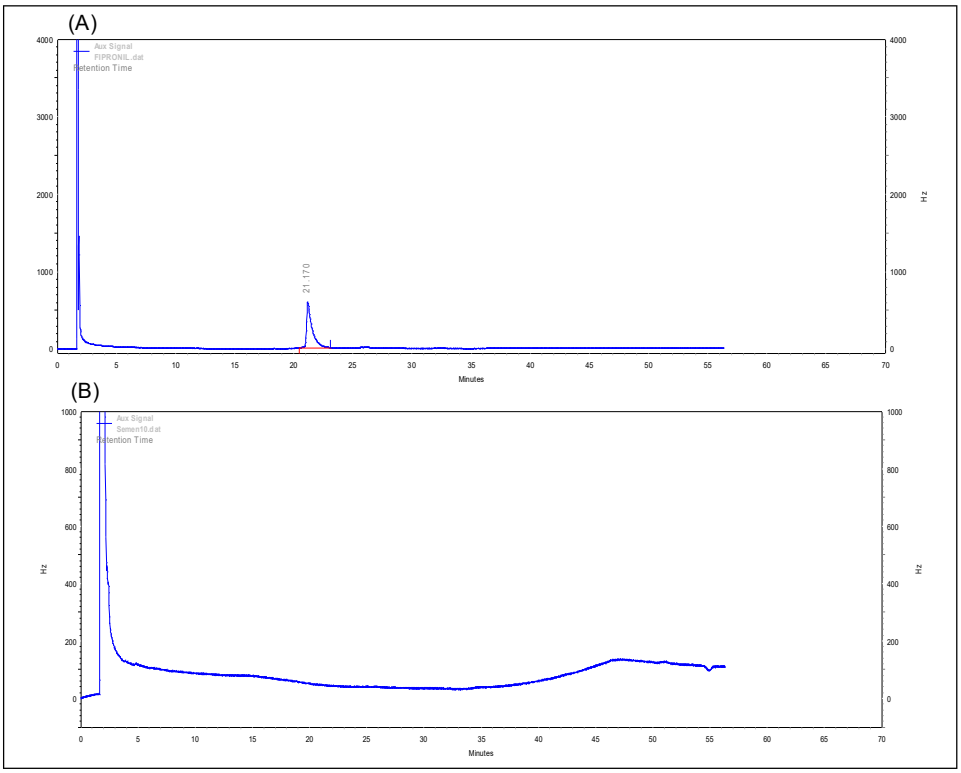


**Fig 3:** Chromatogram of A) Standard (at retention time of 21.17 min.) and B) Semen for fipronil, (showing no peak in semen sample).

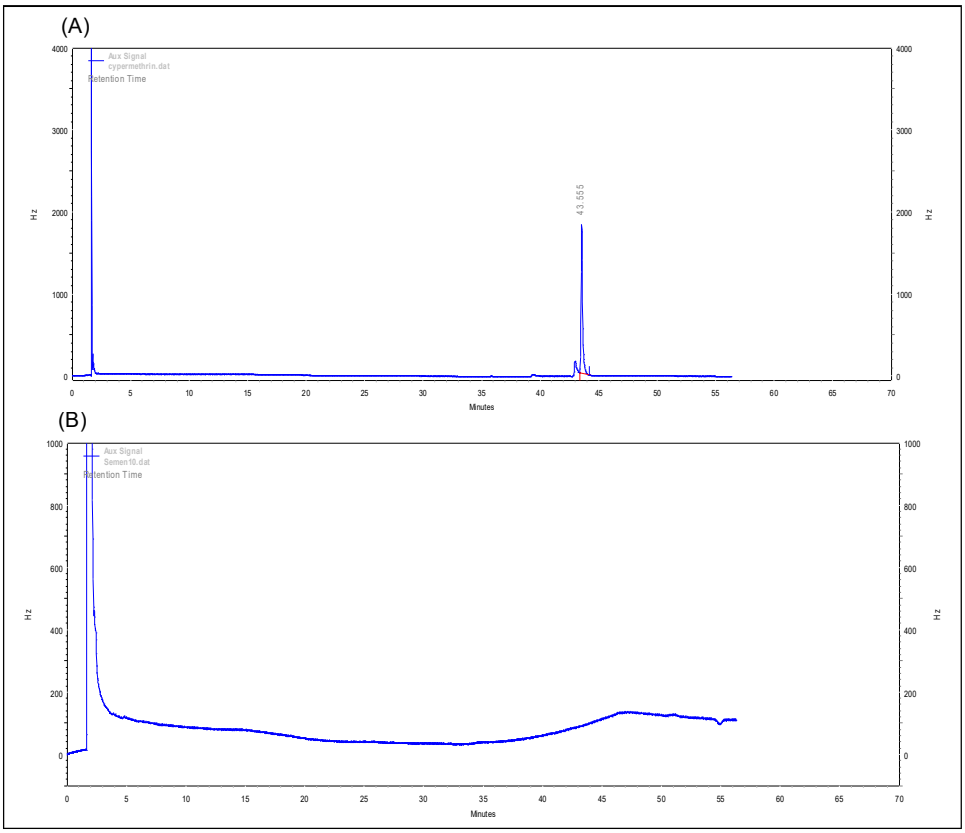


**Fig 4:** Chromatogram of A) Standard (at retention time of 43.555 min.) and B) Semen for cypermethrin (showing no peak in semen sample).

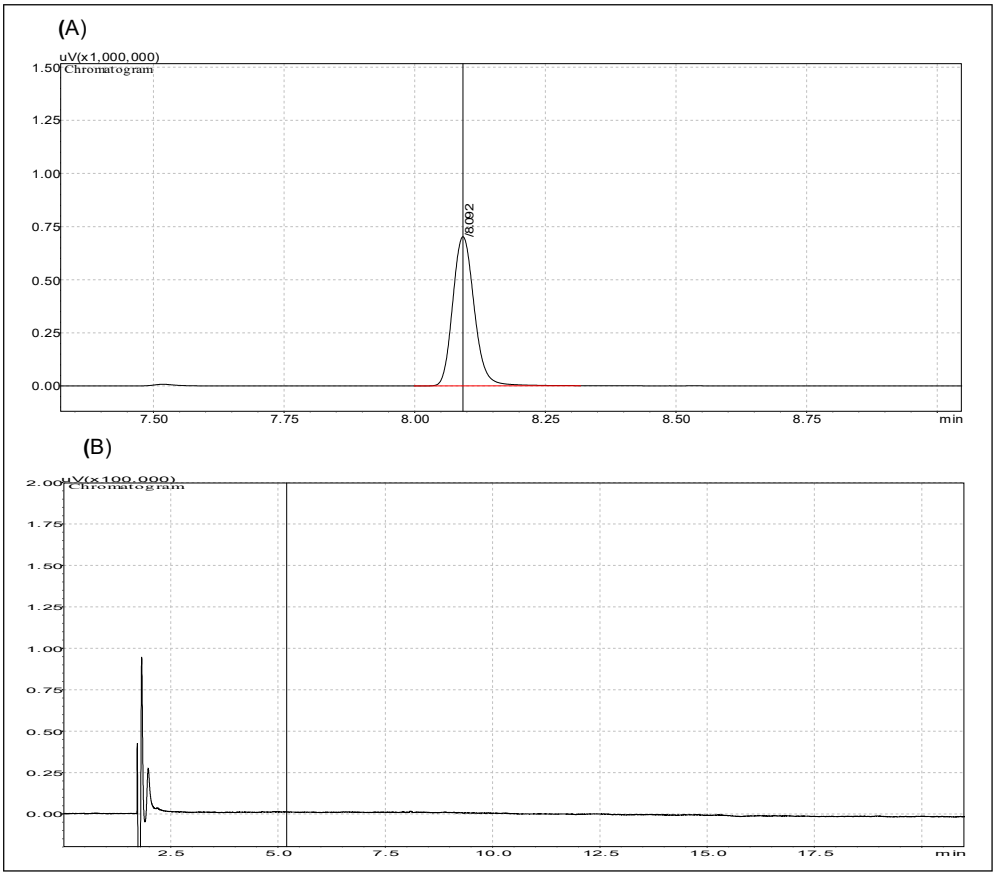


**Fig 5:** Chromatogram of A) Standard (at retention time of 8.092 min.) and B) Semen for parathion (showing no peak in semen sample).

**Viability (%) and total sperm abnormalities (%)**

In present study, viability (%) of sperms were found to be significantly higher ($p<0.05$) in fresh (75.57±5.31) as compared to post thaw (70.17±4.64) semen of bulls. Also, the abnormality (%) of sperms was found to be significantly lower ($p<0.05$) in fresh (13.61±3.71) than post thaw (18.22±3.59) semen bulls. The results of the present study are in slight concurrence with previous study (Vyas *et al.* 1992). They reported that the abnormal spermatozoa rate was 9.58±0.86 initially in fresh semen sample and 14.07±0.87 after thawing in frozen semen sample in HF crossbred bulls. Soren *et al.* (2016) observed 10.74±0.18% major sperm abnormalities in adult healthy Karan Fries bulls and found that they were increased in hot-humid as compared to spring at Animal Breeding Research Centre (ABRC) of National Dairy Research Institute (NDRI), Karnal. The variation in the abnormal spermatozoa rate may be due to season, affection of testis and defects during preparation of slides.

**Membrane integrity (%)**

In present study the HOST (%) was found 67.23±4.76 in fresh semen which decreased to 46.82±3.55 in post thaw semen.The results of the present study for hypo osmotic swelling test are slightly higher than Zubair *et al.* (2013). They reported that the mean sperm positive to HOST was 27% in crossbred semen, 40% in Frisian semen and 47% in Sahiwal semen. Zodinsanga *et al.* (2015) reported that 53.8% of tested bulls exhibited >35% hypo-osmotic swelling response analyzed from frozen-thawed semen of 14 cross-bred and 12 pure bred bulls. The results of present study for HOST are lower than Soren *et al.* (2016). They reported the value for Hypo-osmotic swelling test to be 59.75±0.57 and 64.97±0.84% in the summer and spring seasons, respectively, in Karan Fries bulls from Animal Breeding Research Centre (ABRC) of National Dairy Research Institute (NDRI), Karnal. Chauhan *et al.* (2017) reported HOS test value of 73.76±0.47% Frieswal bulls. The variation in the HOST may be due to variation in percent osmolarity, method of HOST, cryopreservation damages to spermatozoa.

## REFERENCES

Alemu, J., Mohaed, H., Mahendra, P. and Bula, A. (2014). Comparative studies on semen for gross and microscopic abnormalities in bulls at preservice evaluation time. Haryana Vet. 53(2): 135-38.

Angasaria, H.R., Pareek, P.K. and Dati, M. (2002). Seminal characteristics in Holstein Friesian cross breed. Indian Journal of Animal Research. 36(1): 49-51.

Chauhan, H.S., Kumar, R., Kumar, S., Tyagi, S. and Rajkumar (2017). Physical and morphological characteristics of Frieswal bull semen. International Journal of Communication Systems. 5(3): 865-67.

Gopinathan, A., Sivaselvam, S.N., Karthickeyan, S.M.K. and Kulasekar, K. (2016). Effect of body weight and scrotal circumference on semen production traits in crossbred Holsetin Friesian bulls. Indian Journal of Animal Reproduction. 39(1): 24-27.

Guardino, X., Serra, C., Obiols, J., Rosell, M.G., Berenguer, M.J. and López, F. (1996). Determination of DDT and related compounds in blood samples from agricultural workers. Journal of Chromatography A. 719: 141-47.

Hossain, M.E., Khatun, M.M., Islam, M.M. and Miazi, O.F. (2012). Semen characteristics of breeding bulls at the central cattle breeding and dairy farm of Bangladesh. Bangladesh Journal of Animal Science. 41(1): 15-18.

Kamarianos, A., Karamanlis, X., Theodosiadou, E., Goulas, P., Smokovitis, A. (2003a). The presence of environmental pollutants in the semen of farm animals (bull, ram, goat, boar). Reproductive Toxicology. 17: 439-45.

Kanchan and Matharoo, J.S. (2015). Effect of semen colour on seminal characteristics in cattle bulls. Indian Journal of Animal Research 49(1): 146-47.

Kumar, A., Singh, J., Dhaliwal, G.S. and Singh, P. (2008). Incidence and factors associated with poor libido in breeding buffalo bulls. Indian Journal of Animal Sciences. 78: 143-45.

Mathur, A.K., Kumar, S., Prabhakar, J.H., Mandal, D.K., Ingale, H.R. and Jose, J. (2014). Motility of frozen thawed semen after thermal resistance test. Indian Journal of Animal Reproduction. 35(1): 18-20.

Ratnakaran, U., Ghuman, S.P.S., Bedi, J.S. and Gill, J.P.S. (2014). Pesticide residue accumulation in buffalo ovaries: A potential hazard to fertility. Indian Journal of Animal Sciences. 84(7): 741-44.

Shaha, S.P., Alam, M.G.S., Khatun, M. and Ahmed, J.U. (2008). Breeding soundness of stud bulls. The Bangladesh Veterinarian. 25: 51-61.

Smith, S. (1995). Agricultural Recycling of Sewage Sludge and the Environment. Wallingford, UK: CAB International. 207-36.

Soren, S., Singh, S.V. and Kumar, A. (2016). Influence of season on semen quality in karan fries (Tharparkar × Holstein Friesian) bulls. Journal of Animal Research. 6(2): 121-25.

Thullner, F. (1997). Impact of pesticide resistance and network for global pesticide management based on a regional structure. World Animal Health Review. 89: 41-47.

Vyas, S., Greesh, M., Dhami, A.J. and Sahni, K.L. (1992). Studies on the norms and correlations of initial and post thaw seminal attributes of triple crossbred bulls. International Journal of Animal Science. 7: 73-76.

Zama, A.M and Uzumcu, M. (2010). Epigenetic effects of endocrine-disrupting chemicals on female reproduction: An ovarian perspective. Frontiers in Neuroendocrinology. 31: 420-39.

Zodinsanga, V.P., Mavi, S., Ranjna, S., Kumar, A. and Gandotra, V.K. (2015). Relationship between routine analysis/sperm function and fertility tests of cattle bull semen. Asian Journal of Animal Sciences. 9: 37-44.

Zubair, M., Lodhi, L.A. and Ahmad, I. (2013). Hypo osmotic swelling test as screening for evaluation of semen of bull. Journal of Entomology and Zoology Studies. 1(6): 124-28.