Philipp J Vet Anim Sci 2017 43 (2):123-132

RESEARCH NOTE:

EVALUATION OF FROZEN SEMEN STORED IN PROVINCIAL AND FIELD STATIONS IN BICOL REGION, PHILIPPINES

Lourdita A. Llanto

ABSTRACT

This study described the storage, handling and quality of frozen semen samples used in artificial insemination (AI) in cattle and Philippine water buffalo at provincial and field stations in Albay, Camarines Sur and Sorsogon in Bicol region, Philippines. Frozen semen straw samples were analyzed for motility, viability and morphology at the Philippine Carabao Center, Carranglan, Nueva Ecija. Interviewed were three Provincial AI coordinators and 26 AI technicians involved in AI. Frozen semen straws from the National Artificial Breeding Center and Philippine Carabao Center were stored in center tanks at the regional station in Camarines Sur where these are distributed to the different municipalities of Camarines Sur, and the provincial stations (Albay and Sorsogon). In Albay and Sorsogon, center tanks served as storage tanks and "station thawing" was commonly practiced during conduct of AI. In Camarines Sur, AI technicians used field tanks as storage tank. Storage rooms for center and field tanks were spare rooms without air-condition or corners inside offices. Number of handlers of frozen semen who were also trained AI technicians ranged from one to four. Storage of frozen semen samples ranged from few days to one year. Percent post-thaw motility (PTM) did not deteriorate based on subjective evaluation. However, computer-assisted sperm analysis showed 23.53% of samples had <70% PTM. There was highly significant reduction ($p \leq 0.01$) in percent live and normal sperm when data from source was compared with results of laboratory analysis. Percentage abnormal sperm was higher in semen from cattle bulls.

Key words: artificial insemination, cattle semen, thawing, water buffalo

INTRODUCTION

The Unified National Artificial Insemination Program (UNAIP) was created in 2000 to localize the delivery of the artificial insemination (AI) services by the local government units (LGUs). The Dairy Roadmap 2010-2016 recognizes AI as a key component in the development of the dairy industry in the country. In 2012, a total of 90,925 AI services have been provided by the LGUs for cattle, carabaos (Philippine water buffalo), and goats, producing 18,097 calves of improved genetics nationwide (BAI, 2012). The percent share of the National Dairy Authority - assisted dairy projects to total milk production from 2011-2012 was 73.16% of which 52% was from Luzon (NDA, 2012). Three provinces (Albay, Sorsogon and Camarines Sur) of the Bicol region are dairy project areas assisted by NDA out of the total 12 assisted dairy projects in Luzon. In 2011, 97.64% of 254 dairy cattle and 61.85% of 1,295 Philippine water buffalos artificially inseminated in Bicol region were from these dairy areas (DA-RFU V, 2012). This study was a component of a dissertation which aimed to assess the AI service in the three dairy areas of the region. No study has assessed the frozen semen quality in Bicol region. Results of the study could

Bicol University College of Agriculture and Forestry, 4503 Guinobatan, Albay, Philippines (email: lourditallanto@gmail.com; lallanto@bicol-u.edu.ph).

provide useful information on how to improve AI service, thus, increase conception rate which ultimately could help in herd build up and improve dairy production. It is also hoped that the results of the study might be useful for planning purposes of the Livestock Division of the Department of Agriculture in Region V in the implementation of the UNAIP.

This study aimed to evaluate the quality of the frozen semen used in artificial insemination at the provincial and field station levels in Albay, Sorsogon and Camarines Sur provinces of the Bicol region. Storage and handling practices were described.

MATERIALS AND METHODS

Frozen semen straw samples were randomly taken from LN_2 tanks in June 2012 at the provincial and field stations in Camarines Sur, Albay and Sorsogon. Mother or center tanks had a 30-liter LN_2 capacity which can hold 500 frozen semen straws while field LN_2 tanks with 10-liter LN_2 capacity had 50 frozen semen straws since this was the allotment provided to field stations. Frozen semen straw samples were directly taken from the storage sites of the LN_2 tanks. At the Department of Agriculture –Regional Field Unit 5 station, the samples were taken from the mother tank stored in one corner of the office. Samples from Sipocot and Lupi in Camarines Sur, were taken from the field tanks stored in the corner of a spare room and in an air-conditioned office, respectively. In Albay provincial station, the samples were taken from the mother tank stored inside the Albay Dairy Processing Center. Similarly, in Sorsogon provincial and field stations (City Veterinary Office, Sorsogon Dairy Farm and Municipal Agriculture Office-Gubat) the frozen semen straws were taken from mother/center tanks stored in stock room and offices. Samples from these stations were transported in a 10- liter capacity LN_2 field tank to the Philippine Carabao Center – Central Luzon State University (PCC-CLSU) Semen Processing Laboratory in Carranglan, Nueva Ecija. Number of samples depended on the available frozen semen straws in the tank of the stations. As such, there were only a total of 33 frozen semen straws collected from nine (9) stations. All samples were analyzed for sperm motility, viability (percentage of live/dead sperm), and morphology (percentage of normal/abnormal sperm). Data for % post-thaw motility, % live sperm and % normal sperm of the samples prior to distribution to the stations were retrieved from the sources - Philippine Carabao Center (PCC) and National Artificial Breeding Center (NABC) for comparison with the data for the same parameters. Hence, the analysis was conducted by different laboratory technicians for the initial and final data.

Frozen semen samples were submitted to PCC-CLSU Semen Processing Laboratory for motility evaluation using the Computer-Assisted Sperm Analysis (CASA) system and subjective evaluation. The amount of LN_2 in the field tank with 10-liter LN_2 capacity was determined prior to removal of the frozen semen straws to ensure quality of the samples was not compromised due to the long travel distance. The laboratory technician measured the LN_2 in the tank to be 16 cm which was 2 cm above the required 14 cm. Frozen semen samples were thawed at 40°C for 20 sec and analyzed for motility using the Computer Assisted Sperm Analysis (AXiO, Germany) and subjective evaluation. For the subjective evaluation, a small drop of semen sample was placed on each end of a prewarmed microscope slide and covered with a cover slip. The slide was placed in the stage warmer at 39°C and viewed under phase contrast microscope at 200-400x magnification. Subjective evaluation of motility was conducted by only one person who is the experienced laboratory technician of PCC. The guide by Mamuad *et al*. (2005) was used.

Smears from thawed samples were prepared and stained with eosin-nigrosin (1% eosin and 5% nigrosin in 3% sodium citrate solution) to determine percentage of live/dead sperm. Three (3) replications per sample were prepared. Minimum of 300 sperm cells were randomly counted from each smear. Colorimetric detection was used to determine viability of the semen. Dead sperms were fully stained while alive sperms were not. Half-stained sperm cells were considered dead. Percent live sperm was computed.

Abnormalities in the morphology of the sperm were also noted using the same stained samples used for the counting of live/dead sperm. The spermatozoa was classified as normal or as having primary or secondary abnormalities. Primary abnormal spermatozoa were microcephalic head, elongated narrow head, pyriform head, double head, swollen midpiece, dag defect, pathological midpiece, corkscrew midpiece, knobbed sperm, double tail, abaxial midpiece, broken head and underdeveloped sperm. Sperm with abnormalities were recorded and counted. Percentage normal/abnormal sperm was computed.

The t-test was used to determine the difference on semen quality of frozen semen samples from the original source and the station. Percentages and means were computed and descriptive analysis was also used.

RESULTS AND DISCUSSION

The regional station at Camarines Sur which served as the repository of frozen semen straws from PCC and NABC, was headed by the Livestock Division Head who had a personnel assigned as Regional Artificial Insemination Coordinator (RAIC). Every province had a Provincial Artificial Insemination Coordinator (PAIC) and a provincial station except in Camarines Sur where the regional station also served as the provincial station. At the regional station, center or mother tanks and field tanks were kept in one corner of the air conditioned office. Distribution or release of semen and LN_2 was done by the RAIC. In his absence, trained AI technicians of the Livestock division can release frozen semen. As such, the number of handlers of the frozen semen ranged from one to four. Nearby municipalities in Camarines Sur regional station availed of AI services provided by the regional station itself. Distant municipalities such as Sipocot, Ragay, and Lupi would go directly to the regional station to get their LN_2 and frozen semen supply. In the field stations in Camarines Sur such as Sipocot, Lupi and Ragay, frozen semen was stored in 10 liter LN_2 capacity field tanks and brought along during AI service. The mother tank served only as storage for LN_2 and was kept either inside the air-conditioned office or in another room without air condition. According to the AI technicians, this practice allowed them to save on LN_2 gas although they needed to replenish the field tank every week using the LN_2 gas from the mother tank. Handlers of frozen semen were AI technicians themselves. In Sipocot, 4 AI technicians handled frozen semen stored in one field tank while in Lupi and Ragay, 2 AI technicians handled frozen semen.

Albay and Sorsogon provinces got their supply of LN_2 and frozen semen directly from the regional station in Camarines Sur. In Albay, the provincial station is located at the Provincial Veterinary Services Office in Legazpi City near the Provincial Capitol Building. However, the center tank was kept in the stock room of the Albay Dairy Processing Plant in Cabangan, Camalig, Albay. Only one AI technician had access to the center tank and was responsible for the release of the frozen semen straws. The same personnel also served as the AI technician of the province along with the PAIC. Aside from the center tank, there was also one field tank used by the AI technician during conduct of AI.

The provincial station in Sorsogon is located at the Provincial Veterinary Office near the Capitol Building of the province. Both center tank and field tanks were kept inside the stock room with no air conditioning unit. Aside from the PAIC, there were two other AI Technicians assigned to release frozen semen. Sorsogon provincial station did not serve as the distribution center of the province. Offices with mother tanks such as the City Veterinary Office (CVO), Sorsogon Dairy Farm (SDF), and the Municipal Agriculture Office (MAO) in Gubat, got their supply directly from the regional office. The Provincial Veterinary Office (PVO) used its own supply for its own AI service in the province. Three AI technicians handled frozen semen straws. During the conduct of AI, the AI technicians did not use field tanks to transport the frozen semen. Instead they put the semen straw in a small cooler filled with cracked ice and a gel pack. "Station thawing" as they called it, enabled them to save on LN_2 according to the AI technicians. It also lightens the load since they did not have to carry the field tank. Based on the interview, this practice allowed the frozen semen to retain its frozen state for a maximum of 2 hours travel. Hence, it is in reality not really thawing. For CVO, two employees (the AI technician and the livestock inspector) handled frozen semen straws. Semen was stored in the mother tank of the AI center of the city. The AI technician used field tank for his AI service. At SDF, frozen semen was kept at the mother tank inside their office and any of the 3 AI technicians of the agency can get frozen semen straws from the storage tank. Just like the PVO, AI technicians of SDF used "station thawing." The frozen semen straw is placed in an alkaline purifier thermos bottle filled with cracked ice. Hence, the field tank was not used to transport semen straws during AI service. This practice according to the technicians retains the frozen state of the semen even up to 3 hours travel. At the MAO-Gubat office, semen was stored in the mother tank kept inside their office. Two AI technicians handled frozen semen straws. Field tank was used by the AI technician during AI service. In general, storage areas of the AI stations were either spaces in their offices or spare rooms except for the CVO where their storage room was inside the office of the AI center for swine.

All LN_2 tanks whether center or field tanks were kept in vacant spaces inside offices and placed directly on the floor. According to Du Ponte (2007), to ensure maximum holding time of liquid nitrogen, the tank should be stored away from direct sunlight in cool, clean, dry, dust-free, well ventilated place. The tank must not be stored directly on concrete floor but should be elevated on wooden pallet to prevent acids in the concrete from corroding the bottom of the tank.

Handlers of the frozen semen straws were trained AI technicians but the number of handlers ranged from 1 - 4 AI technicians per station. Storage of the frozen semen was variable - from as early as 18 days to as long as 432 days.

Table 1 shows that despite variation in storage and number of handlers, subjective evaluation of the samples showed no significant difference on the initial and final % post-thaw motility. This indicated that storage and handling used did not affect the post-thaw motility of the frozen semen. However, based on more reliable evaluation (computer-assisted sperm analysis), 70% post-thaw motility was noted. There were 4 (23.53%) samples with <70% post-thaw motility, namely: DA-RFU V; SDF, Sorsogon; MAO-Gubat,

Table 1. Sperm motility of frozen semen samples from different stations in the Bicol Region after storage.

Station	Storage conditions	Number of handlers	Sample no.	Length of storage (days)	Mean post-thaw motility (%)		
					Initial[1]	Final[1]	
					SE[2]	SE[2]	CASA[3]
Albay Provincial Veterinary Services	Stock room; no aircon	1	2GPO1089	120	35	35	91.16
			2GPO1050	120	40	40	86.78
Sorsogon Provincial Veterinary Office	Stock room; no aircon	3	2GPO1102	126	40	40	93.33
City Veterinary Office	AI Center Office; with aircon	2	BRA32[4]	120	25	25	74.22
			No Label	30	-	30	76.19
Sorsogon Dairy Farm	Inside office; no aircon	3	BRA32[4]	134	25	25	82.39
			2GPO3005	44	40	40	89.86
			No Label	120	-	20	64.82
Municipal Agriculture Office-Gubat	Inside office; with aircon	2	2GPO6083	30	35	35	86.32
			No Label	130	-	25	67.26
Camarines Sur Department of Agriculture –RFUV	Inside office; with aircon	4	BRY19[4]	432	25	25	64.43
			No Label	30	-	35	83.44
Municipal Agriculture Office – Sipocot	Corner of training hall; no aircon	4	2GPO6083	120	33	33	90.14
			BRA32[4]	127	25	25	75.33
			No Label	120	-	32.5	68.53
Municipal Agriculture Office - Lupi	Inside office; with aircon	2	BRA32[4]	124	25	25	71.78
			No Label	18	-	37.5	86.76

Sorsogon; and MAO-Sipocot with 64.43%, 64.82%, 67.26%, and 68.53% post-thaw motility, respectively). These stations had more than one handler. Station with only one handler had >70% post-thaw motility (PVS, Albay). In addition, the sample stored longest also had the lowest % post-thaw motility (64.43%). Stroud (2012) reported that several handling events if improperly performed expose frozen semen to temperatures that can lead to cell damage or even death. Damage from each exposure is cumulative and straws inside the tank can also be damaged especially when the semen straw lifted out of the LN_2 tank is returned again. It is possible that the adjacent semen straws could also be affected.

Table 2 shows the morphological evaluation of the frozen semen samples in terms of live and normal sperm. Percent live sperm both for the initial values (data from the sources - PCC and NABC) and the final values (results of laboratory analysis after storage

Table 2. Sperm viability of frozen semen samples from different stations.

Station (No. of Handlers)	Sample no.	Mean Live Sperm ** (%)		Mean Normal Sperm ** (%)	
		Initial[2]	Final[3]	Initial[2]	Final[3]
Albay Provincial Veterinary Services (1)	2GPO1089	98.80 [a]	90.07 [b]	98.80 [a]	85.05 [b]
	2GPO1050	95.34 [a]	89.31 [b]	84.17 [a]	80.15 [b]
Sorsogon Provincial Veterinary Office (3)	2GPO1102	89.94 [a]	83.69 [b]	93.18 [a]	85.38 [b]
City Veterinary Office (2)	BRA32[1]	-	90.26	77.52 [a]	75.82 [b]
	No label	-	90.76	-	76.90 [b]
Sorsogon Dairy Farm (3)	BRA32[1]	-	88.81	77.52 [a]	76.02 [b]
	2GPO3005	89.66 [a]	85.49 [b]	96.55 [a]	84.57 [b]
	No label	-	93.05	-	82.45
Municipal Agriculture Office-Gubat(2)	2GPO6083	97.77 [a]	91.97 [b]	97.21 [a]	80.74 [b]
	No label	-	89.77	-	81.96
Camariner Sur Department of Agriculture –Region 5 (4)	BRY191	-	89.33	83.33 [a]	77.77 [b]
	No label	-	92.30	-	83.00
Municipal Agriculture Office – Sipocot (4)	2GPO6083	95.48 [a]	91.92 [b]	93.05 [a]	84.09 [b]
	BRA32[1]	-	91.06	77.52 [a]	76.51 [b]
	No label	-	91.80	-	85.12
Municipal Agriculture Office – Lupi (2)	BRA32[1]	-	89.32	77.52 [a]	75.22 [b]
	No label	-	91.64	-	80.28

** highly significant ($p \leq 0.01$); [1] cattle species (Note: no superscript on the sample number means it is from Philippine water buffalo bull); [2] - the data from the source (Philippine Carabao Center and National Artificial Breeding Center); [3] - as analyzed of frozen semen at the stations) was relatively high in all stations irrespective of storage and number of handlers. This implies that the frozen semen kept in the stations were still of good quality and fertile. Frozen semen with >70% live sperm are considered of good quality (RCA, 2004) while sperm with questionable fertility has <50% live sperm (Vale, 2004). However, statistical analysis showed a highly significant difference ($p \leq 0.01$) between the initial and the final percentage live sperm of the samples. This indicates that despite the fact that the level for good quality sperm has been maintained even after handling and storage in the different stations, the reduction in the % live sperm was significantly different. Although the laboratory analysis for the initial live sperm and final live sperm was conducted by different individuals, this result could be a cause for concern since reduction in percent live sperm after storage could lead to a reduction in the number of fertile sperm.

On the other hand, % normal sperm from Philippine water buffalo bulls in all stations, irrespective of storage and number of handlers, was above the 75% minimum allowable normal sperm, implying good quality sperm. However, the normal sperm of cattle bulls were much lower (75.22% - 77.77%) and very near the borderline value of 75%. Statistical analysis showed there was a highly significant reduction in the normal sperm of the samples after storage and handling. This suggests that there is the possibility of reduction or lowering of number of normal sperm with prolonged storage and handling.

Table 3 shows the percentage of abnormal sperm found in the frozen semen samples at the different stations. Samples from cattle bulls had a higher % abnormal sperm irrespective of number of handlers and storage than Philippine water buffalo bulls (22.23%-24.78% vs. 14.62 – 20.40%). It must be noted that the same samples had already a relatively low initial % normal sperm compared to Philippine water buffalo bulls (Table 2). Good quality semen should have < 20% abnormal sperm (Vale, 2010; RCA, 2004) but Mamuad *et al.* (2005) recommended allowable values of 25%-30% abnormal sperm.

Of the two types of abnormalities, primary abnormality is considered more severe since this is caused by faulty spermatogenesis (Mamuad *et al.*, 2005). The data shows that the % primary abnormality in all stations was generally lower than the secondary abnormality. Primary and secondary abnormalities in bull sperm are shown in Figure 1.

PVS, Albay and Sorsogon Dairy Farm (SDF), had the lowest incidence of primary abnormality wherein 3 out of the 14 types of primary abnormalities or 21.43% were observed in semen samples from their tanks. These were microcephalic head, knobbed sperm and broken head for Albay and microcephalic head, elongated head, double tail, abnormal midpiece, and undeveloped sperm in Sorsogon. There were more types of primary abnormalities (6 - 9) or an incidence ranging from 42.86 - 64.29%, found in semen samples stored at MAO-Sipocot; MAO-Lupi; and DA-RFU V, all in Camarines Sur province. Others like MAO-Gubat and PVO (all in Sorsogon) had 4 - 5 types of primary abnormalities in the semen samples. Samples from CVO, Sorsogon had 2-6 defects.

PVO Sorsogon had the lowest number of 4 types or an incidence of 50% of secondary abnormalities followed by PVS Albay with 5-6 types. SDF, Sorsogon had 5-7 types of secondary abnormalities (62.50-87.50%). Samples from MAO-Gubat, Sorsogon, MAO_Lupi, Camarines Sur and DA-RFU V, Camarines Sur had 7-8 types of abnormalities

Table 3. Abnormalities of semen samples after storage at provincial/field stations.

Station (No. of Handlers)	Sample no.	Abnormal Sperm (%)	Type of Abnormality (%)		Incidence of Abnormal Sperm/ Sample (%)	
			Primary	Secondary	Primary (n=14)	Secondary (n=8)
Albay Provincial Veterinary Services (1)	2GPO1089	14.95	0.40	14.54	21.43	62.50
	2GPO1050	19.85	0.39	19.46	14.29	75.00
Sorsogon Provincial Veterinary Office (3)	2GPO1102	14.62	1.66	12.96	28.57	50.00
City Veterinary Office (2)	BRA32[1]	24.18	1.22	22.96	42.86	100.00
	No label	20.40	1.00	20.40	14.29	100.00
Sorsogon Dairy Farm (3)	BRA32[1]	23.98	0.59	23.39	21.43	62.50
	2GPO3005	15.43	0.49	14.94	14.29	87.50
	No label	17.55	1.32	16.23	21.43	62.50
Municipal Agriculture Office-Gubat(2)	2GPO6083	19.26	0.67	18.59	35.71	87.50
	No label	10.08	0.38	17.66	28.57	100.00
Camarines Sur Department of Agriculture – Region 5 (4)	BRY19[1]	22.23	1.24	20.99	64.29	100.00
	No label	17.00	0.68	16.32	28.57	87.50
Municipal Agriculture Office – Sipocot (4)	2GPO6083	15.91	1.64	14.27	50.00	100.00
	BRA32[1]	23.49	2.12	14.48	57.14	100.00
	No label	14.88	0.93	13.95	42.86	100.00
Municipal Agriculture Office – Lupi (2)	BRA32[1]	24.78	2.30	22.48	57.14	87.50
	No label	19.72	1.23	18.49	50.00	87.50

[1]cattle species (Note: no superscript on the sample number means it is from Philippine water buffalo bull)

(87.50-100% incidence). Samples from two stations - CVO Sorsogon and MAO-Sipocot, Camarines Sur had all of the 8 types of secondary abnormalities (100% incidence). The most common defects which were found in all 17 samples were free head, midpiece and tail; coiled midpiece/tail; bent midpiece and coiled tail. This was followed by bent tail (94.12%) and proximal droplet (70.59%). The least common were distal proximate

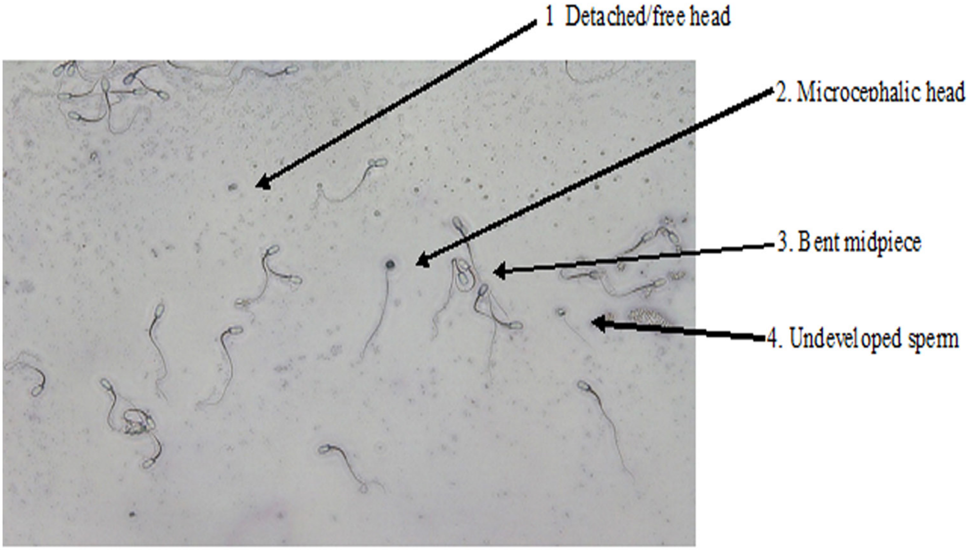

Fig. 1. Abnormalities in Philippine water buffalo bull semen samples (400x magnification); 1) Detached or free head – a type of secondary abnormality wherein the head of sperm is separated from its body; 2) Microcephalic head – a primary abnormality wherein the sperm has a small head which is due to the lesser chromosomal content of the sperm cell than normal; 3) Bent midpiece – a secondary abnormality wherein the midpiece portion of the sperm is bent, and 4) Undeveloped sperm – a primary abnormality in which the sperm is smaller than normal or is not fully developed.

droplet and detached galea capitus (58.82%). The presence of protoplasmic droplet whether proximal or distal is due to short stay of sperm in epididymis. During sperm cell maturation, cytoplasmic droplet migrates down the tail away from the head and drops off at the midpiece. Short stay in epididymis leads to failure for cytoplasmic droplet to completely drop off at the midpiece. Although considered normal, large number of this defect indicates abnormal spermatogenesis or epididymal maturation. Sperm with protoplasmic droplets are still immature and thus, incapable of fertilizing the ova (Vilakazi, 2003; Mamuad *et al.*, 2004; Flowers, 2004).

Proper handling and storage are critical to maintain the quality of the frozen semen. Hence, facilities to house the AI related activities particularly the LN_2 tanks should be established. The storage room for LN_2 tanks must be well-ventilated, preferably air-conditioned, secured and should contain only AI paraphernalia including record books and monitoring forms for inventory purposes. LN_2 tanks must not lie directly on the floor but should be elevated on wooden pallets.

The number of handlers should be limited to one to minimize deterioration of quality of the frozen sperm due to handling. However, if it is inevitable to have more than one handler, they must be trained or retrained on proper storage and handling of frozen semen. They must also be regularly monitored to ensure proper handling procedures are met and that quality of the semen is not compromised.

ACKNOWLEDGMENT

The author is grateful to her adviser; panel members; Livestock Division of DA-RFU V; NABC-BAI; PCC; Nueva Ecija Stock Farm; PAS/CVO/MAO/SDF in Albay, Camarines Sur and Sorsogon; DA-BAR; ETSP-EVP and BUHRDP.

REFERENCES

BAI. 2012. Highlights of accomplishments 2012. Accessed 10 January 2013. www.bai.da.gov.ph.

Cruz LC. 2010 . Recent developments in the buffalo industry of Asia. *Proceedings of the 9th World Buffalo Congress April, 2010*. pp 7-19. Accessed 12 January 2013. www.vet.unne.edu.ar/revista/21-suple-1/00%20invitados.

Flowers WL. 2004. Detailed description of sperm motility/morphology and causes of abnormalities. *Proceedings of Midwest Boar Stud Conference II*. Accessed 4 February 2013. www.bsmc.missouri.edu/2004_proceedings.pdf.

Mamuad FV, Venturina E and Saito H. 2004. *Collection, processing and handling of buffalo semen*. PCC, Central Luzon State University, Science City of Muñoz, Nueva Ecija, Philippines. 75pp.

Mamuad FV, Venturina H, Venturina E, Morcoso R, Atabay E and Kudo K. 2005. *Artificial Insemination Manual for Water Buffaloes*. WBBCIP. JICA-Assisted Project of the PCC and BAI, Philippines.

NDA. 2012. Performance of the dairy industry. Accessed 6 February 2013. www.nda.da.gov.ph/dairyzones.

RCA. 2004. Guidelines and recommendations for improving artificial breeding of cattle and buffaloes in Asia. RCA Working Document. Austria. RCA, IAEA, FAO. Accessed 6 November 2012. www.-naweb.iaea.org/nafa/aph/public/ras-aimar04.pdf.

Stroud B. 2012. Consequences of mishandling frozen semen and embryos. *Proc. Applied Reproductive Strategies in Beef Cattle.* Pp, 191-204. Accessed 22 January 2013. www.appliedreprostrategies.com/pdfs/2012ARSBC-15StroudProceedings.pdf.

Vale WG. 2004. Principles of estrus detection and AI. *Proceedings Pre-Congress Training on the use of reproductive biotechniques in water buffaloes.* Oct. 4-19, 2004. p.18.

Vale WG. 2010. Deep freezing buffalo semen – state of art. *Proceedings of the 9th World Buffalo Congress, April 2010. Pp. 844-855.* Accessed 16 January 2013. www.vet.unne.edu.ar/revista/21-suple-1/10a%20bufalos.pdf.

Vilakazi DM. 2003. Factors affecting the quality of the semen of Artificially Inseminated dairy bulls in South Africa. MS Thesis, Univ. of Pretoria. 98 pp. Accessed 19 November 2012. www.upetd.up.ac.za/thesis/available/etd-0902205-150724.