Storage, Handling, and Distribution of Frozen Equine Semen

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1. Introduction
With the recent acceptance of frozen semen by the American Quarter Horse Association and the American Paint Horse Association, the overall interest and use of this breeding method has increased significantly in recent years. Proper handling and maintenance of semen frozen and stored in liquid nitrogen is critical to the success of artificial insemination with frozen semen. International distribution of frozen semen allows stallion owners access to foreign markets and provides a method for mare owners to access semen from stallions that are the best genetic matches for their mares. The development of the frozen semen industry depends upon high standards of quality control set by the industry and the adoption of fair and equitable breeding contracts that offer guarantees of fertility similar to those offered for cooled or fresh semen contracts.

2. Liquid Nitrogen Storage Systems
The majority of equine frozen semen is stored in aluminum, double-walled, vacuum-sealed liquid nitrogen containers. The inner container wall is surrounded by insulating foil and then enclosed in an outer aluminum wall. The air is evacuated from the space between the two walls and sealed. Access to the inner container is through a long neck tube that is sealed with a styrofoam neck cork. The superior insulating properties of this system allow for the maintenance of liquid nitrogen temperatures throughout the container even when only a few centimeters of liquid is present. For storage of tens of thousands of straws in a single container, many large commercial bull studs utilize large stainless steel storage containers. Both types of containers provide safe, reliable, ultra-low temperature storage for semen samples without relying on electricity or mechanical parts. Several manufacturers produce these containers in a wide variety of capacities and holding times to fit most any need. A list of several popular liquid containers produced by Minnesota Valley Engineering, Inc. along with their specifications is given in Table 1.

<table>
<thead>
<tr>
<th>Liquid Nitrogen Container</th>
<th>Capacity (straws)</th>
<th>Holding Time (days)</th>
<th>Manufacturer</th>
</tr>
</thead>
<tbody>
<tr>
<td>Container A</td>
<td>5,000</td>
<td>30</td>
<td>Minnesota Valley Engineering, Inc.</td>
</tr>
<tr>
<td>Container B</td>
<td>10,000</td>
<td>60</td>
<td>Minnesota Valley Engineering, Inc.</td>
</tr>
</tbody>
</table>

There are two important factors to consider when selecting a liquid nitrogen storage container: storage capacity and holding time. The number of straws or frozen semen that can be stored in a given tank depends upon the size of the straws, the manner in which they are packed (bulk vs. goblets on canes, etc.) and the size and number of canisters within the container. Some canisters are too short to hold 4 or 5 ml “macrotubes” used by some semen freezing facilities. Try to anticipate storage requirements prior to selecting a nitrogen container. The second factor to consider is the availability of liquid nitrogen to refill the containers on a regular schedule. The refill frequency will depend upon the holding time or “working duration” for a given container. The volume of liquid nitrogen determines the holding time that the container holds, the nitrogen evaporation rate, and the frequency with which the container is opened. The nitrogen evaporation rate is directly related to the diameter of the neck opening. A small diameter opening provides longer holding time because of the reduced evaporation rate. However, the smaller opening also limits the
size and number of canisters, thereby reducing storage capacity. Ideally one should select a container that provides the required amount of storage capacity with the longest possible holding time. We have found that the MVE XC 47/11 (or similar model from other manufacturers) offers the best combination of large capacity and nitrogen efficiency for semen stored in our laboratory. While these containers are extremely reliable, occasionally they will lose vacuum and fail. To protect against the possible loss of an entire inventory of semen from a given stallion, it is advisable to have more than a single container so that the inventory from each stallion can be divided among different containers. Also, if a tank loses vacuum and starts to rapidly evaporate nitrogen, you must have another container to transfer the semen into.

3. Maintenance of Liquid Nitrogen Containers

Properly maintained, frozen semen can remain viable for many years. Following some simple guidelines for proper care and handling of liquid nitrogen containers will help prolong the useful life of the containers and ensure that valuable frozen semen is not lost due to tank failure.

1. Keep containers in a well-ventilated, dry room. Nitrogen gas escaping from storage containers is colorless, odorless, and tasteless and the amount that escapes from properly functioning containers is minimal. However, it is remotely possible that if containers were failing and kept in a very small poorly ventilated room, the nitrogen in the air could accumulate, reducing the percentage of oxygen to dangerous levels.

2. Do not place aluminum containers on concrete floors. Concrete flooring promotes corrosion of the aluminum and could lead to tank failure.

3. Keep containers in an area where they can be visually inspected on a daily basis. A container that is rapidly losing vacuum will accumulate frost around the neck. If it is noticed, the semen can be transferred to another container before all of the nitrogen evaporates and the semen is lost.

4. Check liquid levels once per week and record to determine the evaporation rate of each individual container. To check liquid levels, use a dark colored, solid dipstick. Place the dipstick into the container and allow it to freeze then remove the dipstick, and read the liquid level as the lowest part of the u-shaped frost line. All containers will eventually lose some vacuum. By checking levels regularly and recording evaporation rates you can identify those tanks that are becoming inefficient and transfer semen before a problem occurs. Most manufacturers will also refurbish older containers and re-evacuate and seal them to prolong the useful life.

5. Top off containers when liquid level drops to half capacity.

6. Never allow liquid levels to drop below 5 cm. Some manufacturers produce alarm devices that can be fitted to containers and signal when liquid nitrogen drops to dangerous levels.

7. Inspect the neck cork regularly for damage. Damaged corks will result in greater nitrogen evaporation and reduced holding time.

4. Inventory Control

The amount of semen in inventory and the rate at which inventory levels fluctuate determines what type of system works best for documenting frozen semen inventory. In our laboratory we have developed a computerized system, which allows inventory levels to be updated easily as new semen is added or existing semen is removed. The system records all of the pertinent identifying information on the straws as well as package type, post-thaw quality, export status, and container location. This system allows our office to receive a shipment request from a stallion or mare owner and have a shipping container packed and labeled for shipment within the hour. The system also generates transaction summaries and current inventory reports to be sent to clients along with monthly storage bills. Obviously, smaller inventories do not require such an elaborate system. However, some insurance com-

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**Table 1. Selected Chart—MVE Aluminum Liquid Storage Containers**

<table>
<thead>
<tr>
<th>MVE Model</th>
<th>SC 3/3</th>
<th>SC 11/7</th>
<th>SC 20/20</th>
<th>XC 22/5</th>
<th>XC 34/18</th>
<th>XC 47/11</th>
</tr>
</thead>
<tbody>
<tr>
<td>Max. Storage Capacity</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No. of Canisters</td>
<td>6</td>
<td>6</td>
<td>6</td>
<td>6</td>
<td>6</td>
<td>6 (or 10)</td>
</tr>
<tr>
<td>No. 0.5-ml straws (canes)</td>
<td>—</td>
<td>540</td>
<td>540</td>
<td>2,400</td>
<td>2,100</td>
<td>4,500</td>
</tr>
<tr>
<td>No. 0.5-ml straws (bulk)</td>
<td>732</td>
<td>780</td>
<td>780</td>
<td>3,666</td>
<td>3,000</td>
<td>6,216</td>
</tr>
<tr>
<td>No. 5 ml Macrotubes</td>
<td>—</td>
<td>150</td>
<td>150</td>
<td>480</td>
<td>450</td>
<td>990</td>
</tr>
<tr>
<td>Unit Dimensions</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Neck Opening (mm)</td>
<td>2 (51)</td>
<td>2 (51)</td>
<td>2 (51)</td>
<td>3.81 (97)</td>
<td>3.5 (89)</td>
<td>5 (127)</td>
</tr>
<tr>
<td>Canister Height (mm)</td>
<td>5 (127)</td>
<td>11 (279)</td>
<td>11 (279)</td>
<td>11 (279)</td>
<td>11 (279)</td>
<td>11 (279)</td>
</tr>
<tr>
<td>Weight Full, lbs (kg)</td>
<td>14.4 (6.5)</td>
<td>36.6 (16.6)</td>
<td>62.5 (28)</td>
<td>66 (30)</td>
<td>96 (43.5)</td>
<td>120 (54.6)</td>
</tr>
<tr>
<td>Performance</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>LN Capacity (l)</td>
<td>3.6</td>
<td>11</td>
<td>20.5</td>
<td>22.4</td>
<td>34.8</td>
<td>47.4</td>
</tr>
<tr>
<td>Working Duration (d)</td>
<td>19</td>
<td>35</td>
<td>137</td>
<td>40</td>
<td>123</td>
<td>76</td>
</tr>
</tbody>
</table>
companies will require a properly documented semen inventory and nitrogen level log to qualify for coverage. Numerous manual systems have been used. One system that works well involves index cards for each ejaculate of semen stored in a given tank. As straws are removed, the index card is updated so that a running inventory of that batch is readily accessible. When a new batch is frozen, a new card is added to the file for that tank. Each card contains all the pertinent information regarding that batch of semen.

5. Working Safely with Liquid Nitrogen

At atmospheric pressure, nitrogen in liquid form boils at a temperature of $-196^\circ$C ($-320^\circ$F). Obviously, anything this cold is dangerous and precautions must be taken to avoid serious injury when handling it. Additionally, liquid nitrogen expands tremendously upon vaporizing. One liter of liquid nitrogen expands to nearly 25 cubic feet of nitrogen gas. The following precautions are recommended when working with liquid nitrogen.

1. Do not allow liquid nitrogen to contact skin. This can cause extreme frostbite, freezing human tissue almost instantaneously.
2. Do not allow objects cooled by liquid nitrogen to touch bare skin. These objects may stick to the skin and tear away tissue when the object is removed. Always use forceps or tongs to remove items from storage containers.
3. Do not seal containers tightly. Nitrogen storage containers must be adequately vented in order to prevent a build up of gas pressure that could severely damage or even burst the container. Icing or frost could prevent proper venting and neck tubes should be checked for such obstructions.
4. Avoid spilling or splashing nitrogen when transferring from container to container. Use of phase separators on filling hoses from pressurized tanks will minimize splashing of nitrogen during container refilling.
5. Never use a hollow tube or rod to measure liquid levels as the gasification and expansion of the rapidly warming liquid inside the tube will force liquid to spurt from the top of the tube.
6. Liquid nitrogen should be disposed of only in outdoor areas by pouring slowly onto the ground where it can evaporate into the open air.

6. Frozen Semen Distribution

Shipping Frozen Semen

Care should be taken when transferring frozen semen from storage to shipping containers. Exposure of frozen semen to room temperature should be no more than a few seconds; therefore, this should be performed with shipping and storage containers placed side by side. Always work within the neck of the storage container, below the frost line, while locating, identifying, and grasping straws. Use a pre-cooled hemostat or tweezers and transfer the straws quickly one by one to the shipping container. Be careful not to drop the straws into the bottom of the canister as they are extremely brittle at this temperature and could easily crack. Lower the straws into the canister slowly and then the canister back into the shipping container. Repeat this procedure until all the straws are loaded.

As equine semen is frozen in a variety of package types, the number and size of straws to be shipped will vary from laboratory to laboratory and stallion to stallion. If 0.5 ml straws are used, they may be stored in bulk; loosely held in large goblets with diameters close to the size of the canister inside the shipping container. Alternatively, they may be stored 4 or 5 straws per small goblet placed onto aluminum “canes.” This is the primary storage method for bovine semen. Large volume straws, typically 4 or 5 ml volume “macrotubes” are also commonly used for equine semen and are placed directly into canisters without goblets. In either case, cotton should be placed around the brittle straws to prevent them from hitting the side of the canister or goblet during shipping.

In an effort to monitor temperature histories inside shipping or storage containers, we routinely use special indicator ampules. Two glass ampules containing colored liquids are mounted onto an aluminum cane. The two colored liquids have different melting points. The blue liquid melts at $-100^\circ$C and if melted indicates a potentially damaging temperature rise at some time inside the tank. The red liquid melts at $-55^\circ$C, a temperature which may damage frozen semen and indicate that semen quality has possibly been compromised. To use, the liquid in the glass ampules is frozen in liquid nitrogen while holding the can upside down (i.e., the liquid is frozen in the wide base section of the ampule). Once the ampules are cooled completely, the cane is inverted and placed inside the canister of the shipping container. If at anytime during shipment the temperature inside the canister rises to dangerous levels, one or both of the liquids will melt moving it into the bottom of the ampule. Even if the tank is refilled, the ampule will be “tripped,” indicating a history of elevated temperature and alerting the recipient of a possible problem.

Shipping Containers

Although some distributors transport frozen semen in small liquid storage containers, shipping frozen semen is best accomplished using containers specifically designed for transport. Chart–MVE and Taylor Wharton both produce vapor shipping cartons that safely and economically maintain liquid nitrogen temperatures for small quantities of semen over a one to three week time period. The inner cavities of all vapor shippers are surrounded by layers of hydrophobic absorbent material which when charged contains the liquid nitrogen in vapor phase and maintains an inner cavity temperature of less than $-150^\circ$C. Table 2 lists the various vapor series
shippers currently available from Chart-MVE. Note the range of capacities and holding times available. A very important consideration is the "full weight" of these shippers as most express mail services are quite expensive.

The advantages of using vapor shippers are: 1) safety and 2) non-hazardous classification. Liquid shippers which are laid on their side or tipped over during transport will lose all liquid nitrogen and warm rapidly, destroying valuable semen and perhaps costing stallion owners thousands of dollars. Also, spilled liquid nitrogen is a safety hazard and could cause serious shipping delays and possible injury to handlers. Because of this, liquid shippers must be classified as containing hazardous materials, which requires special paperwork and increases shipping costs. Vapor shippers, on the other hand, contain no "liquid" nitrogen per se. The nitrogen vapor absorbed into the containers insulating layer provides the coolant. Hence, vapor shippers are often referred to as "dry shippers." Although holding time of vapor shippers is reduced if laid on their sides, some vapor will remain absorbed and keep the semen safely frozen for a short period of time. No liquid nitrogen capacity means no hazardous materials classification and fewer shipping restrictions. Vapor shippers are generally lighter than liquid storage tanks and are therefore also less expensive to ship.

Chart–MVE manufactures a "mushroom" shaped protective shipping carton for all sizes of shipping tanks. The carton is base-wide with a rounded top that reduces the likelihood of the tank tipping over or being loaded upside down or on its side. The hard molded plastic carton also helps prevent tank damage due to normal shipping use.

One disadvantage of using vapor shippers may be unique to the equine AI industry. Unlike the bovine industry, mare owners and veterinarians do not generally own nitrogen storage tanks. Therefore, it is often necessary for the inseminating veterinarians to keep the semen for some time after shipping in the transport tank. Our distribution contracts allow for mare owners to keep shipping tanks for 10 days after arrival to function as short-term storage tanks during the mare's estrous. Some vapor shippers do not have holding times long enough to allow this. In cases where nitrogen storage is not readily available, we ship semen in small liquid shippers (SC11/7) or 2- and 3-wk vapor shippers (SC 4/2V, SC 4/3V). To increase the safety of liquid shippers, Chart–MVE has developed "vapor canisters" which contain the same absorbent material as is found in vapor shippers. When shipping a small amount of semen in a liquid tank (SC 11/7, for example), one canister could contain the semen and several of the other 5 canister slots can be loaded with vapor canisters. The vapor canisters provide the security of having a few days holding time if the tank is tipped and liquid nitrogen is lost. This is ample time for the tank to be refilled or for the semen to be transferred to another storage tank. A different approach to the above problem is to use a vapor shipper that has been fully charged without dumping out the excess liquid nitrogen. In our experience, this extends the holding time of an SC 4/3 V vapor shipper for 3–5 d.

Most equine frozen semen is shipped using express mail services such as Federal Express and UPS. Before attempting to ship liquid nitrogen containers with any carrier, contact the company and obtain the instructions for properly completing appropriate paperwork as well as instructions for packaging and labeling the containers. Overlooking a small detail on the paperwork or a missing orientation arrow can cause delays in shipment or rejection of the package for delivery.

When shipping frozen semen it is critical for distributors to provide a detailed accounting of semen inventory as well as thorough, clear instructions on thawing, handling, and insemination. All too often, semen is shipped with little or no instruction on its application. Different freezing techniques require specific thawing techniques and the instructions included with the semen should be followed precisely. The freezing organization is in the best position to know which thawing technique and insemination protocol yields the best results for semen from a particular stallion or for semen frozen by a
particular technique. In general, 0.5-ml straws are thawed by immersion in a 37°C water bath for ≥30 sec. When multiple straws are used per insemination dose, all straws should be thawed, dried, and opened so that the contents can be emptied into a sterile pre-warmed container and drawn into an insemination pipette. Some laboratories recommend thawing 0.5-ml straws in a 75°C water bath for exactly 7 sec. Obviously, the timing for this protocol is critical, as exposing the straws to such extreme temperatures for 1 or 2 sec too long will damage the spermatozoa. Semen frozen in large straws (2.5- to 5.0-ml volume) is typically thawed at 50°C for 40 to 45 sec. Once thawed the semen should be inseminated into the mare immediately.

7. Quality Control for Frozen Semen

While no specific standards for frozen semen quality exist in the equine industry, there are some generally accepted guidelines that are adhered to by most of the larger organizations involved in commercial freezing. Methodology to assess sperm quality is the topic of another presentation in this session; therefore it will not be discussed here. Most commercially distributed semen contains between 600 million and 1 billion total sperm per dose, although in France an insemination dose of 400 million total sperm is used. It is generally accepted that thawed semen should contain ≥30% progressively motile sperm and >200 million progressively motile sperm per dose. In our laboratory, semen is frozen to contain 800 million to 1 billion sperm per dose. The total sperm per dose varies depending upon the stallion’s initial semen quality (motility and morphology) and the results of initial test freeze evaluations. Our goal is to provide ≥300 million progressively motile sperm per dose after thawing.

Stallion semen may contain bacteria and viruses that are potential pathogens. Bacteria and viruses survive very well through the cryopreservation process. Extenders for semen freezing should contain antibiotics that prevent the proliferation of bacteria that may be in the semen. Periodic culture of thawed semen from every stallion should be performed to ensure that the antibiotics used are effective in controlling growth of pathogenic bacteria. Furthermore, stallions should be tested for the presence of viruses such as equine viral arteritis virus prior to semen freezing.

8. Exporting Frozen Semen

Frozen semen allows stallion owners access to international markets for the sale of breedings. It also provides mare owners with the ability to choose a stallion that is the best genetic match for their mare without the geographical limitations of fresh or cooled semen breeding. Semen for export must be frozen under strict regulations set by the importing countries. It is important for veterinarians and stallion owners to be aware of these regulations to determine if an international distribution program is appropriate for their situation. Semen for export must be collected and frozen at a facility inspected regularly and certified by the USDA as having met the strict requirements set by the various importing countries. Member countries of the European Union, Australia, and New Zealand all have established regulations and USDA approval requirements for facilities processing semen for export. Both Australia and New Zealand have residency requirements for donor stallions that requires them to enter the collection facility 2–4 wk prior to the start of collections, during which time a number of health tests must be performed. The European Union provides an option for non-resident stallions that allows for semen from a stallion entering the collection facility to be collected and frozen immediately upon entry. The semen must be held at the facility for a mandatory 30-day quarantine period after the last collection during which time the donor stallion must undergo a number of tests to determine health status. The semen may only be exported after the 30-day period and only after all testing is completed and negative results obtained.

Although the details of the collection center requirements differ slightly between various importing countries there are some common general requirements that are listed below.

1. The center must be under the direct supervision of an accredited veterinarian.
2. The center must be inspected biannually by a USDA, APHIS, VS official veterinarian.
3. Donor stallions must have no direct contact with non-center horses.
4. The laboratory for processing semen must be separate from stabling and collection areas.
5. There must be a separate locked area for secure storage of frozen semen.
6. The collection area must be constructed of materials that can be readily disinfected.
7. Detailed records of all center activities must be kept. These include movement of all animals into and out of the center, center activities, testing dates and semen collection, and storage records.
8. New or disinfected collection equipment, storage, and shipping tanks must be used.
9. Egg yolk used in semen extenders must come from flocks of chickens certified free of a number of avian diseases (SPF).
10. Each dose of frozen semen must be permanently labeled with identification of the donor stallion, collection center, and date of collection.

Disease testing of donor stallions and the role of transported semen in the transmission of venereal disease is the subject of another presentation at this meeting and therefore will not be discussed here.

9. Frozen Semen Marketing and Breeding Contract Considerations

One of the main objections to the use of frozen semen by mare owners is that the costs to manage mares for insemination with frozen semen are prohibitive.
Often, frozen semen is purchased “by the dose” with no guarantees and veterinarians are asked to inseminate mares only once during estrous. It has been demonstrated\(^1,2\) that frozen-thawed spermatozoa from some stallions do not bind normally to the mare’s oviductal epithelium, thus reducing the ability of the mare to establish sperm reserves within the reproductive tract. These reserves ensure that viable sperm are present in the oviduct at the time of ovulation when insemination precedes ovulation. Numerous studies have examined the relationship between timing of insemination and fertility.\(^3–5\)

For frozen semen, fertility was reduced when insemination occurred more than 24 h before ovulation or 12 h after. It is generally recommended that frozen stallion semen be inseminated within 12 h prior to or within 8 h after ovulation. The perceived shortened life-span for frozen–thawed stallion spermatozoa within the mares reproductive tract combined with the “by the dose, no guarantee” system of marketing semen has led to the practice of 3 to 4 time per day examinations of mares inseminated with frozen semen. Veterinarians often advise mare owners exploring the use of frozen semen that “around the clock” ultrasound examinations must be performed on mares in the periovulatory period if reasonable pregnancy rates are to be expected. Mare owners are then discouraged from using frozen semen because of the costs associated with such a management scheme. Although it is a widely held belief among practitioners and breeders in North America, there is no evidence in the literature to support the theory that mares inseminated with frozen semen must be inseminated within 6–8 h prior to ovulation to achieve acceptable pregnancy rates. Vidament et al.\(^6\) reported a per-cycle pregnancy rate of 54% for 446 mares inseminated with frozen semen in France. These mares were examined only once daily during estrus and inseminated daily after detection of a 35-mm follicle. These pregnancy rates compare favorably with those reported by Barbachini for mares on a very intense management scheme.\(^7\) After detection of a 35-mm follicle and IV injection of 2000 IU hCG, these mares were examined ultrasonographically every 4 to 6 h until ovulation was detected. The mares were then inseminated with a single dose of frozen semen. The per-cycle and seasonal pregnancy rates for 559 mares were 41.3% and 76.7%, respectively. Obviously, the mares bred in France required more doses per pregnancy than those in the Barbachini report, but the reduced veterinary costs incurred in the management of the mares should offset the increased cost of semen used. The availability of more than one insemination dose per cycle may allow the use of a much simpler, less costly insemination scheme for mares bred with frozen semen.

Stallion owners should be encouraged to formulate breeding contracts for frozen semen that are fair and equitable for the mare owners. The “buy by the dose, no guarantee” approach to marketing frozen semen is counterproductive to developing the frozen semen market. By driving up mare management costs and not providing any guarantees of fertility, this system leaves owners of open mares with a very negative attitude towards the technology. Frozen semen AI is just another method of breeding, like transported-cooled semen AI, fresh semen AI, or natural service. Each method has its advantages and disadvantages. Regardless of the method used, the mare owner is contracting with the stallion owner to produce a foal. This should be viewed as the common goal between the parties. In the bovine AI industry semen is sold by the dose. This system works well for bovine frozen semen because frozen semen of young bulls is adequately fertility-tested, and bulls with poor fertility are generally culled from production. In the equine industry, it may take years to inseminate enough mares with frozen semen from a given stallion before a statistically significant indication of fertility is obtained. We encourage stallion owners to do test inseminations of frozen semen to establish pregnancies before marketing semen to the public. However, many owners do not have access to the mares or are unwilling to use the frozen semen for this purpose. Until a better system for fertility testing frozen semen from stallions is available, stallion owners should be encouraged to offer the same types of guarantees with frozen semen that they would for any other type of breeding method. Furthermore, a contract that provides for at least two inseminations per cycle for up to three or four cycles per season would be more in line with cooled semen contracts and would divide the responsibility for a successful breeding more equally between the stallion and mare owners.

References
8. MVE, Inc. Bloomington, MN 55434-2500.

Proceedings of the Annual Convention of the AAEP 2001

AAEP PROCEEDINGS / Vol. 47 / 2001 301