DEVELOPMENT OF A COMMERCIAL EMBRYO FREEZING PROTOCOL FOR THE CONSERVATION, TRANSPORTATION AND IMPORTATION OF PIG GENETICS
2E-105

Report prepared for the Co-operative Research Centre for an Internationally Competitive Pork Industry

By

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Executive Summary

The ability to import and export pig genetics as cryopreserved embryos would be of immense value to the Australian pig industry. Currently there is no acceptable protocol to allow this. The aim of this project was to develop such a protocol. In order to do this, the following milestones were addressed:

Milestone 1: Improve current culture medium by examining various modifications made in pig as well as other species

Initial experiments investigated various handling media. These showed that the best medium was one developed in our laboratory specifically for pig embryos. A commercially available medium was found to be suitable for the short term storage, handling and cryopreservation of the embryos, but not for medium to long term storage.

Milestone 2: Examine the suitability of Cryologic Block Vitrification method for freezing pig embryos.

Two cryopreservation protocols integrated with the Cryologic Vitrification Method (CVM) were compared. Both produced similar in vitro survival rates post warming (97 to 100% survival) but one was selected because it was faster to perform and technically simpler, both important considerations if the protocol is to be used commercially.

Milestone 3: Develop sanitary protocols for the handling, culture, freezing and transfer of pig embryos which meet the requirements of the International Society for Embryo Transfer.

The method selected in milestone 2 was modified to conform to with protocols developed by the International Society of Embryo Transfer for the international transfer of embryos. These protocols are designed to minimize disease transmission and are used by countries and regulatory authorities around the world. Specifically, to overcome possible contamination via the liquid nitrogen embryos are stored in, we developed a suitable container for the long term storage of embryos. Several containers were tested and one was selected that demonstrated a very low chance of leaking when submerged in liquid nitrogen, is inexpensive, readily available and simple to use. Furthermore, its use did not affect the success of the vitrification process. We then incorporated an embryo washing protocol using a non animal derived trypsin to minimize disease transmission and showed that this did not affect embryo survival post warming.

Milestone 4: Determine the overall efficiency of the method developed by transferring cryopreserved embryos to recipient animals.

The vitrification protocol including all the modifications was then used to cryopreserve and then recover porcine blastocysts which were then transferred into six pseudopregnant recipients (average 26 cryopreserved embryos transferred per recipient). Of these six, five maintained pregnancy and farrowed a total of 26 piglets (average litter size 5.2). This is an excellent pregnancy rate and an adequate litter size for the embryo transfer of cryopreserved embryos.

In summary, this project has resulted in a porcine embryo cryopreservation protocol that complies with the IETS requirements and produces acceptable results. It is reasonably simple to perform and does not require expensive equipment or complex laboratory facilities. It could be used for the importing or exporting of pig embryos with no further modification. However, the regulatory framework by which pig embryos may be imported into and exported from Australia has not yet been worked out.

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1. Introduction

The ability to import and export pig genetics would be of immense value to the Australian pig industry. Currently, the only viable methods of doing this are by using collected semen (which carries only half the desired genetics) or transporting whole animals. Both these methods are associated with the real risk of transmitting disease. However, movement of genetics by embryo transfer has a very low risk of disease transmission. In order to make the international movement of embryos practical, embryos will need to be cryopreserved. Cryopreserved embryos are the preferred method for importing and exporting sheep, cattle and goat genetics into and out of Australia. However, this method is not yet available for importing or exporting pig embryos. This is due to two reasons; primarily, there is as yet no appropriate cryopreservation protocol and secondarily, the regulatory framework for importing and exporting embryos has yet to be worked through and approved by AQIS.

Historically, pig embryos have been problematic to cryopreserve due to the large amount of lipid they contain which is thought to be chemically or physically altered during the cryopreservation process, becoming toxic. However porcine embryos approximately four or more days old appeared to survive cryopreservation better than earlier stages, possibly due to the amount of lipid decreasing as the embryo develops (Nagashima et al., 1992). Over the last 15 years a cryopreservation technique called vitrification has been shown to be successful for porcine embryo cryopreservation (Bethelot et al., 2000; Cameron et al., 2000; Beebe et al., 2002; Cuello et al., 2004). Vitrification is a method by which the embryos are cryopreserved in the absence of ice (which can damage cells) usually by high concentrations of cryoprotectant and rapid cooling. All the molecules in the solution and embryo are 'frozen' in place forming what is known as a glass.

More recently, several technologies have been developed that have greatly increased the success of porcine embryo cryopreservation; the two most relevant of these are minimum volume vitrification and solid surface vitrification (Berthelot et al., 2000; Cameron et al.,2004, Lindemans et al., 2004). It was found that the faster the embryos could be cooled down to the storage temperature (liquid nitrogen -198^{o}C), the better the embryo survival. Both of these technologies achieve this. An Australian company, Cryologic Pty. Ltd., has produced a vitrification system, the Cryologic Vitrification System (CVM), that incorporates both of these technologies and this system has improved bovine embryo cryopreservation (Lindemans et al., 2004) but has not been extensively tested with pig embryos. Furthermore, there are several vitrification protocols that achieve very high survival rates after warming (up to 100%) and acceptable results after transfer to recipient sows (Berthelot et al., 2000; Cameron et al., 2004). These protocols need to be integrated with the Cryologic Vitrification System (CVM), compared and one selected for further development.

Pig embryos are most likely to survive cryopreservation after approximately 4.5 to 5 days or later after fertilization (late morulae to blastocyst stage). However, if the embryos are collected later than day 5 the zona pellucida can rupture. An intact zona pellucida is a requirement for minimum disease transmission risk and a breach in it would make the embryo unsuitable for export. This means there is a relatively small 'window' of embryonic development that is ideal to collect and cryopreserve the embryos. Embryos that are collected late may not have an intact zona pellucida but those collected early have to be cultured in vitro until reaching a stage suitable for cryopreservation. It is obviously preferable to culture early

embryos than have to discard embryos that are not zona intact. However, in vitro culture requires media that allow minimal embryonic degradation during the culture period. The ideal method of in vitro culture is for the medium to be bicarbonate buffered, similar to natural buffer systems. However this requires the embryos be cultured in an atmosphere of 5% CO_2 in order to maintain the correct pH and to do this requires expensive incubators and infrastructure. An alternative is for the medium to be HEPES buffered, which can maintain pH in air and the embryos can then be kept in a smaller portable incubator. While the embryos will generally grow in this medium, the growth will not be as good as in bicarbonate buffered media. It is necessary to investigate several media and holding conditions to determine if embryos can be collected earlier than ideal and held until a suitable stage for cryopreservation.

For any protocol to be used for the import and export of cryopreserved embryos, it must comply with the protocols published by the International Embryo Transfer Society (IETS). These protocols are provided to minimize the risk of disease transmission from embryo transfer and are usually required to be followed by government regulatory bodies. These requirements can be broken down into two categories. The first relates to selecting appropriate embryos. Suitable embryos must have a robust and intact zona pellucida (which provides a partial barrier to infection) and have no adherent matter on the zona pellucida which may present an infection risk. The second relates to processing or 'washing' the embryos after they have been selected but prior to cryopreservation. Washing the embryos involves at least 10 changes of medium including at least two washes in trypsin for at least 30 seconds each. Trypsin washes have been shown to reduce the number of or inactivate pathogens bound to the zona pellucida.

There were two modifications that were needed so that any vitrification protocol complies with these guidelines. The first was to introduce embryo 'washing' prior to cryopreservation. The second was to prevent the embryos or the medium containing them ever coming into direct contact with the liquid nitrogen used to vitrify or store them. There has been some evidence that such contact may be a contamination risk.

The overall aim of this project was to develop a method for freezing pig embryos using vitrification and which meets IETS requirements for importing (or exporting) pig embryos into Australia. This was done in a step wise manner by addressing the following milestones:

1 Improve current culture medium by examining various modifications made in pig as well as other species

2 Examine the suitability of Cryologic Block Vitrification method for freezing pig embryos.

3 Develop sanitary protocols for the handling, culture, freezing and transfer of pig embryos which meet the requirements of the International Society for Embryo Transfer.

4 Determine the overall efficiency of the method developed by transferring frozen thawed embryos to recipient animals

2. Methodology

General Methods

Embryo collection

All the experiments in this project required in vivo produced embryos and these embryos were collected surgically. The donor sow or gilts were given 20 mg Altrenogest (Regumate) for at least 16 days. On the second day after the last dose of altrenogest, the donors were given 1000 IU eCG (Folligon; Intervet Aust. Pty. Ltd.) followed by 750 IU hCG (Chorulon; Intervet Aust. Pty. Ltd.) approximately 80 h later. The donors were artificially inseminated or naturally mated twice during the time of standing estrus, commencing approximately 24 h after hCG treatment. Embryos were collected surgically on the morning of day 5 (day 0 being the onset of estrus). Uterine horns were flushed with 50 mLs of Dulbecco's phosphate buffered saline containing 2% heat inactivated FBS and the flushings collected in a sterile 50 mL tube. The tube was emptied into a sterile 90 mm petri-dish and the contents searched for embryos under a stereomicroscope. Viable embryos were recovered, washed twice in a modified HEPES buffered NCSU23 medium, placed into 3 mLs of the same medium containing 10% FBS in 5 mL tubes and transported from the embryo collection location to the laboratory in a temperature controlled incubator set at 38.5^{o}C.

***In Vitro* Culture**

If required, embryos were cultured post warming in a modified NSCU23 medium containing MEM essential and non-essential amino acids (Beebe et al., 2007) in 50 µL droplets under mineral oil in a humidified atmosphere of 5% CO_2, 5% O_2 and the balance N2 at 38.5^{o}C.

Statistical Analysis

Morphological data were analysed using Chi square or Fisher's exact test. Cell number data were analysed by ANOVA. A p value of less than 0.05 was considered significant.

Milestone 1: Improve current culture medium by examining various modifications made in pig as well as other species

Two basic media were tested. One is commercially available, Medium 199 (M199), and the other was modified NCSU23 (not commercially available), a culture medium created specifically for pig embryos and modified in our laboratory to improve embryo development (Beebe et al., 2007). Both of these basic media come in two variants, HEPES-buffered (which can maintain its pH while exposed to air) and a bicarbonate-buffered version (which require an atmosphere of 5% CO2 in order to maintain correct pH). The media also contained Australian-sourced fetal bovine serum (20% for M199 and 10% for NSCU23) which is standard for culturing embryo of the stages we were collecting. Embryos were collected from donor sows and gilts four days after standing estrus (usually morulae) and were cultured in the various media for 48h in order to compare embryonic survival between the media. The embryos cultured in the HEPES-buffered media were kept in a small portable incubator at 38.5^{o}C and the embryos cultured in the bicarbonate-buffered media were kept in an atmosphere of 5%, CO_2, 5% O_2,

balance N_2 at 38.5°C in large incubator. An extended time in culture was chosen so that the detrimental effects of the culture medium would become apparent, something which might not be detectable after a short period of culture. At the completion of the culture period all surviving embryos were fixed in ethanol and stained with a nuclear dye that allowed counting of the number of cells comprising the embryo.

Milestone 2: Cryologic Block using closed container examined.
The first part of this milestone was selecting the most appropriate vitrification protocol. The CVM was integrated into the protocols and they were compared. The protocols are outlined in Table 1 and 2. After warming, the embryos were cultured in modified NCSU23 medium + 10% FBS for 48 h and survival assessed.

Table 1 - Protocol 1 (EG/DMSO) used for vitrification and warming

Vitrification	Warming
• 1 min M199/FBS[a] • 1 min M199/FBS • 3 min M199/FBS + 7.5% ethylene glycol + 7.5% DMSO[b] • 1 min M199/FBS + 17% ethylene glycol + 17% DMSO + 0.4 M sucrose • Vitrify using CVM[c]	• 1 min M199/FBS + 0.13 M sucrose • 5 min M199/FBS + 0.13 M sucrose • 5 min M199/FBS + 0.075 M sucrose • 5 min M199/FBS

[a] M199/FBS; Medium 199 + 20% heat inactivated fetal bovine serum
[b] DMSO; dimethyl sulfoxide, [c] CVM; Cryologic Vitrification Method

Table 2 - Protocol 2 (EG/PVP) used for vitrification and warming

Vitrification[a,b]	Warming[a]
• 20 min incubation with cytochalasin B in NCSU23 • Centrifuge for 12 min at 13000 x g • 5 min 2 M ethylene glycol at 25°C • Brief wash in 8 M ethylene glycol + 7% PVP at 25°C. • Vitrify using CVM[c]	• Warm in 1M sucrose • 5 min 0.2 M sucrose • 5 min 0.1 M sucrose • Extensive wash

[a] All media is made up with HEPES-NCSU23 at ~38.5°C unless otherwise specified.
[b] All vitrification media contains 7.5 µg/mL cytochalasin B.
[c] CVM: Cryologic Vitrification Method

Cryologic Vitrification Method
The CVM consists of a metal block (Figure 1B) and specially designed storage devices called Fibreplugs™ (Figure 1A). The nylon Fibreplug is made up of a fine hook attached to a handle by a fine thread. The hook is very small and holds about 3 µL (Figure 1C). In use, the metal block is partially immersed in liquid nitrogen and allowed to cool completely down to approximately -198°C. After the vitrification steps described in Table 1 and 2 were completed the embryos were

placed on the hook in approximately 3 µL of the final vitrification medium and the droplet of medium touched to the surface of the cooled metal block. The direct contact between the droplet and the metal allows for very rapid cooling without direct contact with the liquid nitrogen. The Fibreplug was then covered with a cooled plastic sleeve (Figure 1A),immersed in liquid nitrogen and stored.

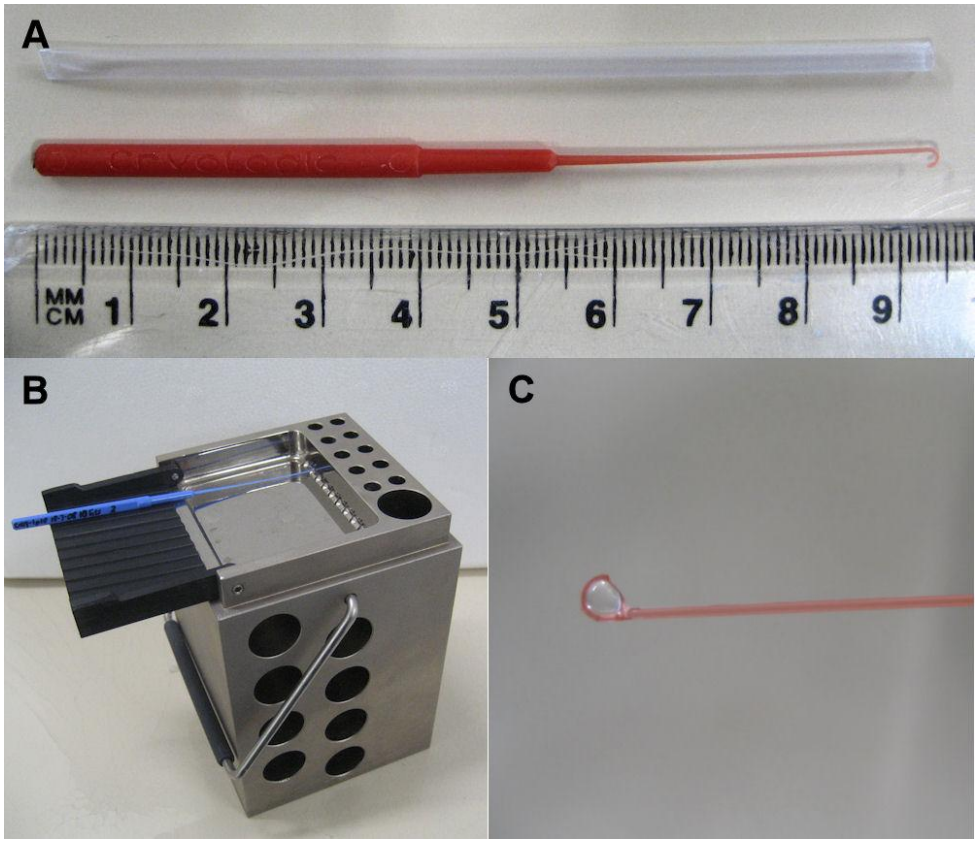

Figure 1 - A: CVM Fibreplug and sleeve, B: CVM block, C: hook on end of fibreplug with 3 µL medium containing embryos.

Liquid Nitrogen Tight (Sealed) Storage Container

As previously mentioned, a requirement for an IETS compliant vitrification protocol is that the embryos and the medium in which the embryos are cryopreserved never make direct contact with the liquid nitrogen used to vitrify or store the embryos. While the CVM system prevents contact between the embryos and the liquid nitrogen during the vitrification process, the sleeve that covers the Fibreplug does not provide a tight enough seal to prevent liquid nitrogen seeping in and contaminating the vitrified droplet containing the embryos while the straw is immersed in liquid nitrogen during storage. Therefore, the Fibreplug needs to be sealed inside a liquid nitrogen proof container. Several containers were tested to see if they could overcome this. The containers examined were sealed, stored under liquid nitrogen for at least a week and then examined for the presence of any liquid nitrogen inside them. After a container was found that didn't show seal failure, embryos were vitrified and stored in them, then warmed and cultured and survival assessed.

Milestone 3: Develop sanitary protocols for the handling, culture, freezing and transfer of pig embryos.
As previously mentioned, the IETS has published protocols for the preparation and handling of in vivo collected embryos which are designed to minimize the risk of disease transmission. The first protocol that needs to be integrated into the cryopreservation protocol is the embryo washes including two trypsin (a protease enzyme) washes. However, the standard trypsin is made from overseas sourced pig pancreas tissue and as such is unacceptable for in vivo use in Australia. There is an alternative, TrypLE Express (Invtrogen Corp), produced using a recombinant enzyme and is free from animal components. As such it is acceptable for use for in vivo in Australia. However, TrypLE Express' effects on porcine embryos are unknown.

The IETS protocol used was as follows:

- 5 x wash in embryo holding medium with at least a 1:100 dilution,
- 2 x 30-45 sec washes in TripLE Express (for a total of 60-90 sec exposure),
- 5 x wash in embryo holding medium with at least a 1:100 dilution,
- Cryopreserve embryos.

In vivo collected late morulae and early blastocysts were collected, washed as above, vitrified and warmed and their survival compared to embryos that were cryopreserved without the IETS compliant wash.

Milestone 4: Transfer of frozen thawed embryos to recipient animals
The final milestone involved transferring frozen thawed embryos to recipient animals to determine the efficiency of the method developed. Early blastocysts were collected and then washed using the IETS protocol described above. They were then vitrified using the CVM method and the fibreplugs sealed into pre-cooled outer straws which were then stored in liquid nitrogen for a period of at least 2 weeks, but normally longer. The embryos were warmed in groups of approximately 25 and the zonae pelluidae removed by exposing the embryos to Acidic Tyrodes solution for approximately 20 sec. The zona free embryos were then extensively washed and loaded into a portable incubator and transported to site of the embryo transfer surgery. The embryos were transferred surgically into the uterus of recipient gilts near the uterine-oviduct junction. The recipient gilts were synchronized as described for the donor gilts but the embryos were transferred on day 4 (day 0 being the onset of estrus), that is an asynchrony of -24 h compared to the embryos. Successful establishment of pregnancy was determined by ultrasound and the pregnant pigs were allowed to farrow naturally.

3. Outcomes

Milestone 1: Improve current culture medium by examining various modifications made in pig as well as other species.

Embryo survival after culture for 48 h in the various media tested is shown in Table 3 and the number of cells in the surviving embryos after 48 h is shown in Table 4.

Table 3 - Embryo survival in various culture media

Medium[a]	Total Embryos Cultured	Survival After 24h (%)	Survival after 48h (%)
NCSU23	35 early morulae	35 (100)	32 (91)[b]
H-NCSU23	38 early morulae	38 (100)	32 (84)[b]
M199/FBS	16 early morulae	14 (88)	0 (0)[c]
H-M199/FBS	17 early morulae	17 (100)	1 (6)[c]

[a] NCSU23, modified NCSU23; H-NSCU23, hepes-buffered modified NCSU23; M199/FBS, Medium 199 + 20% FBS; H-M199/FBS, hepes-buffered Medium 199 + 20% FBS.

[b, c] Values in the same column with different superscripts are significantly different (Fishers exact test). No superscripts means there are no significant differences.

Table 4 - Cell number of surviving embryos after culture in various media

Medium[a]	Cell Number ± S.E.M.
NCSU23	38.3 ± 1.5[b]
H-NCSU23	27.3 ± 1.9[c]
M199/FBS	13.2 ± 1.1[d]
H-M199/FBS	14.6 ± 1.1[d]

[a] NCSU23, modified NCSU23; H-NSCU23, hepes-buffered modified NCSU23; M199/FBS, Medium 199 + 20% FBS; H-M199/FBS, hepes-buffered Medium 199 + 20% FBS.

[b, c, d] Values in the same column with different superscripts are significantly different (AVOVA with Tukey's multiple comparison test post-hoc).

The results of this experiment demonstrated that embryos survived in modified NCSU23 only. Embryo survival in HEPES-buffered modified NCSU23 was similar modified NCSU23, although the blastocyst cell number was lower. This suggests that embryos could be held in a small portable incubator in this medium for a moderate period of time without too much loss in quality. Embryonic development in the commercially available, M199, was very poor with the embryos not surviving the 48 h culture period. However, embryo survival after 24 h in the M199 based media was ok suggesting that this medium would be suitable for the short term

holding of pig embryos, but at some loss of quality. This result also suggests that M199 with FBS would be fine for the short term storage of embryos (several hours) which would be the usual time between embryo collection and cryopreservation.

Milestone 2: Cryologic Block using closed contain examined

Initially, the two vitrification protocols with the CVM system were compared. The results are shown in table 5.

Table 5 - Embryo survival after vitrification and warming using two protocols and the CVM system

Protocol	Total Blastocysts Cryopreserved	Survival After 24h (%)	Survival after 48h (%)
1 (EG/DMSO)	31	31 (100)	31 (100)
2 (EG/PVP)	33	33 (100)	32 (97)

There were no significant differences

Embryo survival after vitrification and warming was similar in both treatment groups. Survival was very high demonstrating the successful integration of the CVM with the two protocols. Although both protocols showed similar embryo survival, protocol 1 was chosen for continued development as it was technically simpler and uses media that is commercially available.

For a sealed storage container, a variety of cryovials were tested initially. These had Teflon washers as part of the lid which were supposed to seal the vial. However, after a week in LN_2 all the tested vials contained or were full of LN_2. Next tested were 5 ml sperm cryopreservation storage straws. These were heat sealed at one end and tested for leaks before use. The other end could be sealed with a plastic sealing ball which was designed for that purpose. Although the manufacturers of the straw and sealing ball could not guarantee the seal against leakage, I found that after 30 tests only one had leaked. Furthermore, when the large straw is stored correctly in liquid nitrogen, it will try to float but is held in place by a cap placed on the storage cane. The end of the sealed straw with the sealing ball ends up in the air bubble contained in the covering cap (see Figure 2B). Therefore, when stored properly the ball seal has virtually no chance of leaking as it is not in contact with the liquid nitrogen. The sealed straw (with the CVM fibreplug inside) and the storage cane are shown in Figure 2.

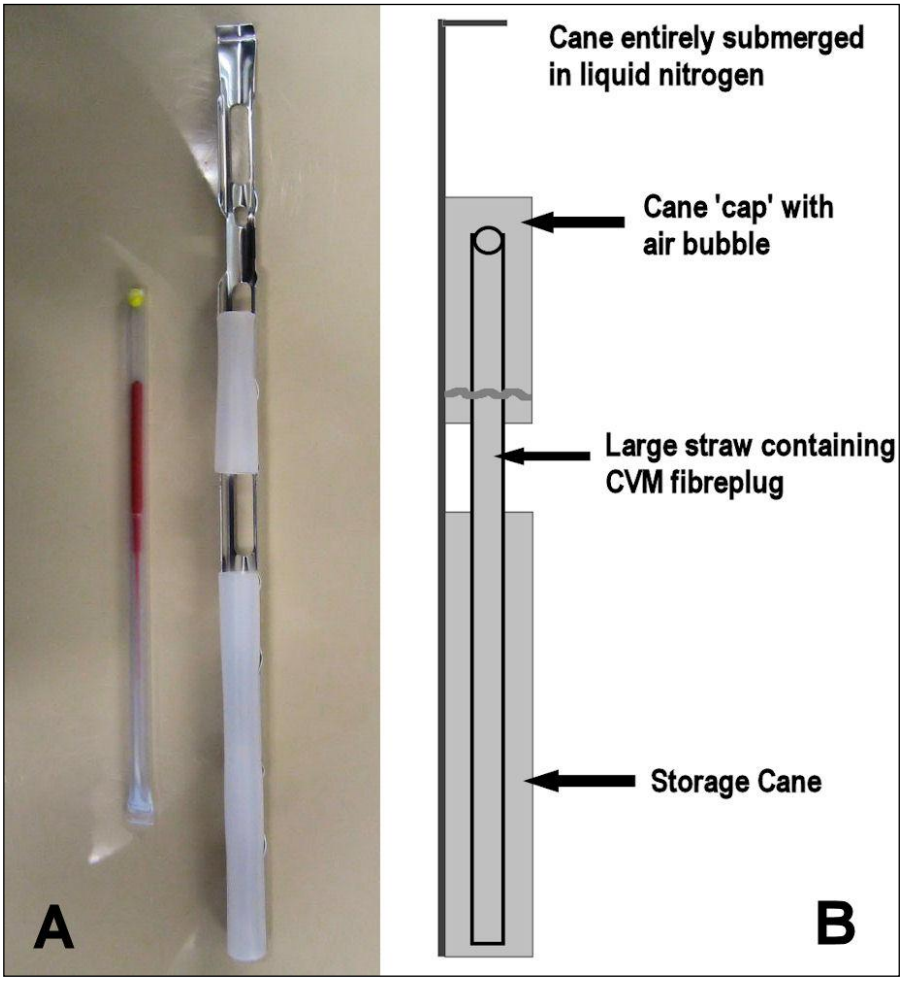

Figure 2 - A: CVM fibreplug sealed inside outer straw and storage cane. Note that the larger straw has the CVM fibreplug inside it, is heat sealed at one end and is sealed with a yellow sealing ball at the other end. B: diagram of the sealed straw during storage. The end of the straw sealed with the ball is inside an air bubble trapped in the 'cap' of the storage cane.

Sealing the CVM fibreplug with the vitrified embryos inside the larger straw and then recovering the embryos from the double straw storage adds considerably to the handling of the embryos at a critical stage when they could be easily thawed accidently. As such it was important to test the protocol with the sealed straw and compare it to the protocol without the sealed straw. The results are shown in Table 6.

Table 6 - Embryo survival in vitro after vitrification in a sealed or unsealed storage container

Storage Container	Total Blastocysts Cryopreserved	Survival After 24h (%)	Survival after 48h (%)
1 Unsealed	57	46 (81)	44 (77)
2 Sealed	59	49 (83)	44 (75)

There were no significant differences

The inclusion of the sealed container in the vitrification protocol had no effect on the in vitro survival of vitrified and warmed blastocysts.

Milestone 3: Develop sanitary protocols for the handling, culture, freezing and transfer of pig embryos.

As mentioned previously, the IETS has published protocols for the preparation of embryos prior to cryopreservation that minimize the risk of transmitting disease. The effect of the IETS washing protocol, including two 30 sec TripLE Express washes, is shown in Table 7. There was no statistically significant difference between the two treatment groups.

Table 7 - Embryo survival in vitro after vitrification and warming including IETS washing protocol

Treatment	Total Blastocysts Cryopreserved	Survival After 24h (%)	Survival after 48h (%)
1 Normal Wash	23	12 (52)	12 (52)
2 IETS wash with TripLE	23	17 (74)	17 (74)

There were no significant differences

These results suggest that the IETS wash protocol and TripLE Express does not impair embryonic survival after cryopreservation.

Milestone 4: Transfer of frozen thawed embryos to recipient animals

The result of the in vivo embryo survival study is shown in Table 8. A total of 151 embryos were transferred into 6 recipient gilts (average of 25 embryos per recipient), of which 5 became pregnant (83% pregnancy rate) and farrowed a total of 26 piglets (15 males, 11 females, average litter size 5.2).

Table 8. Piglets produced after transfer of cryopreserved pig embryos.

Recipient	Blastocysts Transferred	Litter
1	27	Not pregnant
2	26	4 males, 1 female
3	26	1 male, 1 female
4	25	5 males, 3 females
5	24	3 males, 1 female
6	23	2 male, 5 female

The pregnancy rate of 83% is excellent for embryo transfer and the litter size is acceptable but slightly small compared to previously published studies. One factor that may have contributed to this was the recipient. We used gilts that had cycled approximately three times but otherwise their reproductive status was unknown. The best results obtained after embryo transfer is by using second or third parity sows whose fertility is demonstrated. However these are unlikely to be available in practice, except in cases where the embryos are of extremely high value. Unseasonably hot weather which occurred while these transfers were being performed may also have contributed to the low litter size.

4. Application of Research

The present study has developed a relatively efficient, inexpensive and simple method for cryopreservation of pig embryos which meets current IETS requirements. This method results in acceptable pregnancy rates and litter sizes and as such could be used commercially to import, export or conserve pig genetics. However, this protocol requires sign off by the appropriate regulatory authorities such as AQIS before it can be used commercially.

5. Conclusion

In conclusion we have developed a commercially viable method for importing, conserving and exporting pig genetics. Using this method breeding companies, piggeries and producers now have access to pig genetics worldwide.

6. Recommendations

As a result of the outcomes in this study the following recommendations have been made:

Based on the outcomes of this study we would recommend that Pork CRC give consideration to obtaining AQIS approval to use the protocol developed in this study.

7. References

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