

Importation of in vitro-produced *Bubalus bubalis* embryos from Italy into the United States: A case report

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Abstract

On December 19, 2005, 14 in vitro-fertilized water buffalo (*Bubalus bubalis*) embryos, which had been cryopreserved by vitrification, were thawed and transferred into *B. bubalis* recipients in California. The embryos had been produced in Italy, following transvaginal oocyte pickup (TVOPU), with subsequent in vitro maturation, insemination, and culture. This case study relates our experience in meeting the regulatory criteria, established by the Animal Import/Export Office of the USDA-Animal and Plant Health Inspection Service (USDA-APHIS), in order to successfully import these embryos into the USA.

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1. Introduction

From the earliest days of the commercial bovine ET industry in the 1970s, ET promised to be a means of introducing new germ lines across international boundaries [1,2]. Concurrently, leaders in the ET field acknowledged the potential risk of simultaneously introducing foreign animal diseases [1–4]. Advances that have brought about a partial realization of the potential for international movement include improvements in diagnostic sensitivity and specificity of tests applied to potential donors [5]. In addition, improvements in cryopreservation of embryos, standardization

of hygienic procedures for embryo handling, and research that has documented the general safety of international ET, have all contributed to international movement of embryos [6–12].

The agency in the USA responsible for overseeing import and export of animals and animal products (including embryos) is the Animal Import/Export Office of the USDA-Animal and Plant Health Inspection Service (USDA-APHIS). All permits for importation of embryos and semen must be issued by this office. This paper discusses the major criteria which had to be met in order to import “new germ lines” of water buffalo (*Bubalus bubalis*) into the USA from Italy.

2. Signalment and history

A private entrepreneur with an interest in establishing a *B. bubalis* dairy industry in California contacted

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the School of Veterinary Medicine, University of California at Davis. His first request was for assistance with importation of live water buffalo from the Caserta region of Italy. When told that such importation would be impossible (see Section 2.1), he decided to pursue a program of embryo importation, with the goal of producing highly selected Italian Mediterranean buffalo, and breeding this “founder group” with imported semen from progeny-tested bulls in Italy.

2.1. Obstacles to importation

2.1.1. Live animal importation

Following the European outbreaks of Bovine Spongiform Encephalopathy (BSE) in the 1980s and Foot and Mouth Disease (FMD) in 2001–2002, importation of live ruminants was essentially prohibited, with the exception that Mexican and Canadian cattle could be brought into the USA for fattening and/or slaughter. The Harry S. Truman animal quarantine facility (close to the coast of Florida) was deactivated, and in any case, had not been fully utilized because of the high expense importers had to bear in order to keep animals at this facility [13]. As an example, importation of 500 Boer goats from South Africa was estimated to cost \$2000 per goat (\$1M total) for the quarantine alone, excluding all testing costs. Importers received permits on a lottery system, and were required to submit a cashier’s check for \$32,000 for each application to the lottery [13].

2.1.2. Other obstacles

Acute animal disease situations in many countries may prohibit the importation of bovids and bovid products into the USA. In addition, non-tariff trade barriers, e.g., where technical or political issues rather than scientifically derived policies make it difficult or impossible to import/export the product in question, can also be obstacles.

2.2. Consortium formation

An informal consortium was formed among the client and faculty members of the veterinary colleges and schools at the University of California, Davis, the University of Florida, and Federico II University (Naples). The roles of the consortium members were as follows: Italian scientists: (a) procurement of permission from Italian buffalo dairymen to collect oocytes from their animals; transvaginal ovum pick-up; IVF procedures; vitrification of blastocysts; procurement of official paperwork from

appropriate Italian government agencies [14]; (b) UC Davis and University of Florida scientists: selection of domestic recipients; procurement of embryo import permit from USDA-APHIS; receipt of transported embryos; transfer of embryos to recipients; (c) client: funding of importation, animal purchase and maintenance, and payments to Italian dairymen.

2.3. Importation considerations

2.3.1. Practical considerations—availability of recipients

The exact number of *B. bubalis* in the US is unknown, but probably amounts to a few thousand head, mostly descendants of two importation waves in the late 1970s (one from Guam and one from Trinidad-Tobago [15]). The buffalo from Guam were swamp types (karyotypes $2n = 48$), whereas those from Trinidad-Tobago were so called “buffalypso” breed, a composite of river type breeds ($2n = 50$) [15,16]. The distribution of karyotypes amongst the current US population is also unknown, but includes river-swamp hybrids ($2n = 49$), which are reported to be fertile and able to carry embryos of either swamp or river buffalo [16]. The largest concentration of potential recipient buffaloes was a Florida herd of >1000 animals. From this farm, the client negotiated direct purchase of 44 animals meeting the following criteria: (a) manifest fertility (i.e. calf at side); (b) involuted and normal reproductive tract (on the basis of transrectal palpation); (c) a cervix that would allow the passage of an ET pipette; and (d) dairy character, as evidenced by udder conformation, frame type, and to a limited extent, by disposition.

2.3.2. USDA-APHIS requirements, and resources for meeting them

The import protocols of the USDA APHIS animal import-export office are based on recommendations from World Organization for Animal Health (OIE), and from the International Embryo Transfer Society (IETS), as well as on comments from the public. Excellent sources for information regarding the disease status of all exporting countries can be found in: the OIE Terrestrial Animal Health Code, which is updated frequently [17]; the OIE World Animal Health Reports, which details animal health status and disease incidence for so-called “A list” and “B list” diseases, country by country [18]; and a quarterly OIE Bulletin, which is published on-line and is available at no charge [19].

Table 1

Actual required paperwork for importation of Italian water buffalo embryos into the USA

Requirement	Provided by	Comments
Health Certificate for each oocyte donor (multiple embryos/donor allowed, but only one embryo/straw. Each straw must have unique ID number and be listed on certificate	USDA APHIS, to be filled out by local/regional veterinarian	Each embryo must be identified on health certificate by sire and dam; on each straw by sire, dam ID; breed; date of collection; name and address of place where collected; unique ID number for each straw
“Premise Statement” re: TB, Brucellosis, and other infectious diseases	Local/regional veterinarian	As of 2005, bluetongue status was not required
Official seal, signed by regional veterinarian	Regional veterinarian	Make appointments well in advance
Verification of all sires/semen used. (Brucella, TB, BLV* status of bull and bull stud) where semen was collected. *Bovine leukemia virus	Local/regional veterinarian	If AI, generally available from semen provider
Importation permit	USDA APHIS, to be filled out by Importer	Original must accompany shipment. Phone: (301) 734-8364 or on the web at http://www.aphis.usda.gov/vs/ncie
Container sealing	Regional veterinarian	USA government seals. Record seal numbers on health certificate. Ship directly (i.e., no stops before USA) in single-purpose container

3. Operational plan (USDA-APHIS protocols)

Based on past experience by one of the authors (M. Drost) that embryo recovery from superovulated water buffaloes was commonly disappointing, the prospects for importing in vivo fertilized embryos were considered poor [20]. Therefore, we were advised to make our first priority the importation of in vitro fertilized embryos generated from oocytes harvested by transvaginal ovum pickup (TVOPU) [21]. In the absence of a specific protocol for *B. bubalis*, our request for import permits was considered under the bovine rules.

For importation from all countries, the USDA-APHIS National Center for Import-Export protocols require: health certificates for each embryo (see Table 1), signed and stamped by an official veterinarian from the exporting country; import permits obtained through USDA APHIS; and a shipping container used solely for the importation requested, and sealed by the official veterinarian in the exporting country. The semen used to breed the donor cow must have been collected from a “donor sire” at an approved AI center, or been provided by natural breeding to a donor sire at an approved ET unit. Interestingly, in the protocol listed on the Electronic Code of Federal Regulations, Part A (for importation of “certain animal embryos and animal semen” from countries free of rinderpest and FMD), no mention is made of the practice of washing the embryos 10 times before freezing or packaging [22]. However, this requirement is present in part B of that document (“Importation from regions where rinderpest or FMD exist”) [23]. We were advised to follow this portion of

the latter protocol, even though we were not importing from a country with FMD or rinderpest. See http://www.oie.int/eng/normes/mcode/code2006_back/en_chapitre_3.3.1.htm, or <http://www.nesholsteins.com/VERMONT/Embryo%20Marketing/Argentina%20Bovine%20Embryo%20Import%20Requirements.1.pdf>. At the time of our negotiations with the USDA, there was no protocol between the US and either Italy or the EC for in vitro-produced bovine or bubaline embryos, although a draft of a proposal for IVF cattle embryos, endorsed by IETS and OIE (see http://www.oie.int/eng/normes/mcode/code2006_back/en_chapitre_3.3.1.htm) had been awaiting approval from both sides for several months.¹ The USDA-APHIS allowed us to operate under the draft proposal regulations, which are available in detail [22].

Proposed amendments that added non-bovine ruminant embryos to the list of importable commodities, even from countries that had FMD and rinderpest, were published in the Federal Record for public comment. Few comments were received, but some of them were incorporated into the new ruling. For those interested in importation from such countries, the most relevant parts of the amendment dictated that, in cases of importation from countries that have FMD, or rinderpest, an APHIS

¹ As of March 2006, an agreement on importation of in vitro-produced cattle embryos is in effect (see http://www.aphis.usda.gov/NCIE/iregs/animals/ee_bov_emb_annex_3_06.pdf). In addition to addressing FMD, rinderpest, etc., this protocol also requires safeguards against possible bluetongue virus infection. It is presumed that USDA will apply this new cattle protocol to water buffalo.

veterinarian must verify the disease status of the exporting country or region within the country, and conduct on-site inspections of the animals, the premises, and the procedures involved in embryo collection for export [22,23]. It was noteworthy that Italy was free of both FMD and rinderpest.

3.1. Details of the operational plan

3.1.1. In vitro project

A contract was drawn up for the delivery of vitrified, in vitro-produced dairy buffalo embryos from females with credible records of milk production. Under this contract, participating Italian dairyman received a small remuneration for each buffalo cow they enrolled in the program, another small sum for each oocyte retrieval session, a higher sum if those oocytes became freezable blastocysts, and considerably more money for any term calves. The purpose of the payment schedule was to encourage the use of fertile animals, while paying farmers for the inconvenience of having a team of oocyte retrievers on their property on a weekly basis. We followed the draft protocol for in vitro-produced bovine embryos (see below).

3.1.2. Bovine in vivo protocol

As mentioned above, under the importation protocols established by USDA, we were required to obtain import permits for bovine embryos (VS Form 17-129); these forms were available on the Internet (<http://www.usa-federal-forms.com/fbf-by-form/239.html>). This protocol was based almost entirely upon the recommendations of the IETS, as adopted by the OIE [24].

3.1.3. Transvaginal ovum pickup site

The ET team in Italy assisted in donor selection of live animals, and performed TVOPU on site, i.e., at private dairies where donors were housed. The procedure was performed once or twice weekly for up to 10 weeks.

3.2. In vivo-produced embryos from superovulated buffalo

As an alternative to the importation plan, in 2003 our client signed a separate contract with a single Italian farm for in vivo-produced frozen embryos from superovulated donors of high genetic merit. As predicted, the donor buffalo did not yield many embryos; after several months, the contracted farm reported that only five embryos had been obtained and

frozen. Concurrently, we had secured our first permit for importation of in vivo-produced embryos (shipping date: mid-May, 2004), but decided to forego importation until such time when a larger number of in vivo-fertilized embryos could be shipped. In October of that same year, we secured another permit, this time for in vitro-produced embryos; unfortunately, we could not complete the required paperwork in time to meet the time period for shipping window, and the permit lapsed without being used.

The original proposal was to transfer frozen (vitrified)-thawed in vitro-produced embryos into Florida recipients, and at 75 d of pregnancy, to transport pregnant recipients to California. However, this was abandoned due to concerns about the effect transcontinental transport of pregnant recipients during the summer. Instead, we decided to transport non-pregnant recipients to California as soon as selection was completed. Anticipating a favorable decision by USDA, we finished selection of recipients in Florida on August 29 and trucked 44 of them to Davis, CA, USA (approximately 4800 km). That selection process was difficult, due to poor facilities and animal behavior.

3.3. Arrival of *B. bubalis* recipients in California

With the recipient buffaloes in the UC Davis California feedlot, we began a year of intensive study of the non-pregnant water buffalo. We soon noted that one of the 44 was a steer (apparently a loading mistake). We conducted daily observations for estrus (30 min every morning, approximately 07:00–07:30). Concurrently, we conditioned the animals to our presence and collected blood for karyotyping. Of the 43 females, readable karyotypes were obtained on 42, which included 28 river type buffalo ($2n = 50$) and 14 river/swamp hybrids ($2n = 49$). One female's karyotype was undetermined.

3.4. First successful importation of IVP *B. bubalis* embryos into USA

An import permit was obtained the following year (2005) and was timed to allow shipment of vitrified IVF buffalo embryos for transfer to California recipients before the end of the buffalo AI breeding season, i.e. before the end of the calendar year. Our Italian colleagues had amassed 53 IVF embryos, vitrified as Quality 1 and 2 blastocysts. A permit requesting their importation on or about November 28 was filed with USDA-APHIS. Several months before this, we had moved 32 recipients from UC Davis to a dairy in Tulare,

California. To facilitate ET on December 19, all cycling females were subjected to an Ovsynch-CIDR protocol (GnRH + insert CIDR on Day 0, give PGF and remove CIDR on Day 7, and give GnRH on Day 9) [25].

A series of difficulties occurred during the shipment process; fortunately, we had requested that embryos be shipped in liquid nitrogen, rather than over nitrogen vapor, in anticipation of the potential of delayed access. We finally were granted permission to take possession of the container of vitrified *B. bubalis* embryos and we immediately transported them from San Francisco, CA, USA to Tulare, CA, USA (350 km) and prepared for the next day's transfers.

4. Embryo transfer and outcome

Of the 27 synchronized females, 13 were deemed suitable for transfer on the basis of an appropriate CL and suitable reproductive tracts (confirmed by transrectal ultrasonography). On Day 15 (Day 9 = day of second GnRH treatment), 12 recipients received one embryo each and one recipient received two embryos. By verbal agreement with USDA-APHIS, each thawed embryo was expelled from its straw into holding medium and quickly examined for morphology and zona pellucida integrity. Holding medium that had been exposed to the embryo was frozen at -196°C for future PCR assays for pathogens. Embryos were immediately reloaded into sterile 0.25 mL straws and non-surgically transferred to the uterine horn ipsilateral to the CL (single embryo transfers) or one embryo into each horn (twin embryo transfer).

A transrectal ultrasonic examination at 30 d of gestation revealed four pregnancies (30.8% pregnancy rate; 28.6% embryo survival rate.), on the basis of identification of fetal heartbeats; by 50 d, however, all four pregnancies had been lost. An additional 30 embryos were subsequently transferred, omitting the step in which embryos were removed from their straws into holding medium, i.e. they were transferred directly to synchronized recipients without observation of morphology. Six pregnancies (20%) were detected at 30 d, but again all recipients that had been pregnant at 30 d were non-pregnant at 50 d. To date, 10 of 44 (22.7%) of the transferred embryos have established early pregnancy but none has advanced to 50 d.

5. Discussion

This report documents the first importation of in vitro-produced *B. bubalis* embryos into the USA. The success of the importation required considerable input

and good will from a consortium of people in both Italy and the USA; it also required frequent communications with USDA-AHIS, whose personnel were instrumental in the success of the importation. Fortunately, they had anticipated the demand for importation of non-bovine ruminants (including water buffalo). Furthermore, they recognized, in research advocated by International Embryo Transfer Society (IETS) and endorsed by the OIE, that ET can be an effective means for importing new genotypes without concurrently importing new diseases.

Although the pregnancy results were disappointing, we were able to address the numerous requirements (regulations and forms) required for importation. The process was difficult, but hardly impossible. The successful importation will facilitate further studies that may allow us to investigate the current problem of late embryonic-early fetal deaths.

The California members of the consortium had the least experience with *B. bubalis*. Management lessons learned included the difficulties associated with estrus detection. As noted in the literature (and in informal discussions with our colleagues), there was almost no homosexual mounting activity. Thus a timed ET program was our best option. For future studies, we have prepared a teaser bull to assist in detection of estrous cows [26].

The reasons for the failure to maintain pregnancy to 50 d are unknown, but are likely to be a cumulative result of many factors, including possible damage to the oocyte/embryo during in vitro maturation, fertilization, embryo culture and vitrification, sub-optimal handling of the embryos at the time of thaw and transfer, and perhaps most likely, sub-fertility or sub-optimal preparation of recipients, including synchronization. Embryo-recipient synchrony is an important factor in ruminant ET, and particularly so in buffalo, where a half-day of asynchrony can substantially reduce pregnancy rates [27]. That 22.7% of embryos survived beyond the time of maternal recognition of pregnancy suggested that the IVF procedures *per se* produced embryos capable of signaling the dam and preventing luteolysis, at least temporarily. Potential causes of late embryonic/early fetal death include a hostile or non-supportive endometrial environment, luteal failure, genetic or early developmental deficiencies of the embryo itself, or unfavorable external environmental influences, including heat stress, and infectious agents.

In retrospect, the effort to import embryos in time for transfer during the 2005 breeding season was probably ill-advised, as it put undue pressure on our colleagues in Italy. For the 2 weeks after we had received an import

permit, there was a great deal of stress associated with coordinating all the details of paperwork, regional veterinarians' inspection and signatures, etc. Because animals from different geographical areas were used as oocyte donors, several regional veterinarians were required to receive, sign, and stamp the documents. Thereafter, the container had to be sealed, and the documents and container transported to a courier service, which delivered the container to the Rome airport.

We propose to repeat the process, without changing any of the IVM-IVF-culture or vitrification procedures, but with younger recipients acquired from local sources. Unfortunately the recipients that were transported were likely not those that had been selected, and included some obviously aged animals, unthrifty animals, and a steer. To minimize the recipient as a cause of reproductive failure, we plan to use young (primiparous or nulliparous) females. We are currently negotiating for more embryos, and have identified potential sources of young recipients.

6. Conclusions

Importation of embryos from any species is an exercise in observance of details; importation of a relatively exotic animal like *B. bubalis*, brings numerous additional details. Careful coordination of scientific, clinical, and regulatory efforts is critical. Our experience working with the USDA-APHIS was very positive, as this agency was very helpful with meeting the requirements for importation.

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