

# A comprehensive microbiological safety approach for agarose encapsulated porcine islets intended for clinical trials

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## Abstract

**Background:** The use of porcine islets to replace insulin-producing islet  $\beta$ -cells, destroyed during the diabetogenic disease process, presents distinct challenges if this option is to become a therapeutic reality for the treatment of type 1 diabetes. These challenges include a thorough evaluation of the microbiological safety of the islets. In this study, we describe a robust porcine islet-screening program that provides a high level of confidence in the microbiological safety of porcine islets suitable for clinical trials.

**Abbreviations:** Viruses: BDV, Borna disease virus; BVDV-1, -2, Bovine viral diarrhoea virus type 1 & 2; PAV, Porcine adenovirus; PCV, Porcine circovirus; PCMV, Porcine cytomegalovirus; PEV, Porcine enterovirus; PERV-A/B/C, Porcine endogenous retrovirus A B C; PHEV, Porcine hemagglutinating encephalomyelitis virus; PHoV, Porcine hokovirus; PLHV-1, -2 -3, Porcine lymphotropic herpesvirus type 1 2 & 3; PPV, Porcine parvovirus; PRRSV, Porcine reproductive & respiratory syndrome virus; PRCV, Porcine respiratory coronavirus; PRotA, Porcine rotavirus A; PRotC, Porcine rotavirus C; PRV, Pseudorabies virus; PTV, Porcine teschovirus; RV, Rabies virus; REO-1, -2 -3, Reovirus type 1 2 & 3; SEOV, Seoul virus; SIV, Swine influenza virus; SNV, Sin Nombre virus; EMCV, Swine encephalomyocarditis virus; HEV, Swine hepatitis E virus; SVDV, Swine vesicular disease virus; SWPOX, Swine pox virus; TTV, Torque tenovirus; TGEV, Transmissible gastroenteritis virus; EEEV, Eastern equine encephalomyelitis virus; VEEV, Venezuelan equine encephalomyelitis virus; WEEV, Western encephalomyelitis virus; VSV-IN, Vesicular stomatitis Indiana virus; VSV-NJ, Vesicular stomatitis New Jersey virus; WNV, West Nile virus.

Cell Lines: BHK-21, Baby hamster kidney cell line; BT, Bovine turbinate cell line; MA104, African green monkey kidney cell line; MARC-145, Monkey kidney cell line; MDCK, Madin Darby canine kidney cell line; MRC-5, Human fetal lung fibroblast cell line; PAM, Pulmonary alveolar macrophages; PK-15, Porcine kidney cell line; PPK, Primary porcine kidney cell line; ST, Swine testicular cell line

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**Methods:** A four-checkpoint program systematically screens the donor herd (Large White – Yorkshire × Landrace F1 hybrid animals), individual sentinel and pancreas donor animals and, critically, the islet macrobeads themselves. Molecular assays screen for more than 30 known viruses, while electron microscopy and in vitro studies are employed to screen for potential new or divergent (emergent) viruses.

**Results:** Of 1207 monthly samples taken from random animals over a 2-year period, only a single positive result for Transmissible gastroenteritis virus was observed, demonstrating the high level of biosecurity maintained in the source herd. Given the lack of clinical signs, positive antibody titers for Porcine reproductive and respiratory syndrome virus, Porcine parvovirus, and Influenza A confirm the efficacy of the herd vaccination program. Porcine respiratory coronavirus was found to be present in the herd, as expected for domestic swine. Tissue homogenate samples from six sentinel and 11 donor animals, over the same 2-year period, were negative for the presence of viruses when co-cultured with six different cell lines from four species. The absence of adventitious viruses in separate islet macrobead preparations produced from 12 individual pancreas donor animals was confirmed using validated molecular (n = 32 viruses), in vitro culture (cells from four species), and transmission electron microscopy assays (200 cell profiles per donor animal) over the same 2-year period. There has been no evidence of viral transmission following the implantation of these same encapsulated and functional porcine islets into non-immunosuppressed diabetic cynomolgus macaques for up to 4 years. Isolated peripheral blood mononuclear cells from all time points were negative for PCV (Type 2), PLHV, PRRSV, PCMV, and PERV-A, PERV-B, and PERV-C by PCR analysis in all six recipient animals.

**Conclusion:** The four-checkpoint program is a robust and reliable method for characterization of the microbiological safety of encapsulated porcine islets intended for clinical trials.

#### KEYWORDS

pancreatic islets, xenotransplantation, zoonoses

## 1 | INTRODUCTION

The ultimate goal for the treatment of type 1 diabetes is the restoration of physiological blood glucose regulation without the requirement for lifelong therapy such as immunosuppression or immunoregulation. There are numerous challenges for the attainment of this goal, and diverse approaches ranging from insulin pumps to gene therapy to stem cell strategies are all being pursued.<sup>1–5</sup>

In another approach, the replacement of lost islet cells through human islet allotransplantation has established the clinical utility of islet grafting.<sup>6,7</sup> Although only 13% of human islet recipients remain insulin-independent at 5 years, the majority of patients (67%) retain some graft function as evidenced by persistent C-peptide secretion.<sup>8</sup> Importantly, islet transplantation largely eliminates hypoglycemic unawareness post-grafting. Further improvement in long-term insulin independence is likely; data from a few select

Other: ATMP, Advanced Therapy Medicinal Products; CBER, Center for Biologicals Evaluation and Research; CFR, Code of Federal Regulations; cGMP, Current Good Manufacturing Practices; CHMP, Committee for Medicinal Products for Human Use; cpe, Cytopathic effect; DPF, Designated pathogen-free; df, Dilution factor; ELISA, Enzyme linked immunosorbent assay; EMA, European Medicines Agency; FDA, Food and Drug Administration; HA, Hemadsorption test; HEPA, High-efficiency particulate arrestance; HI, Hemagglutination inhibition; IEQ, Islet equivalent; an islet of diameter 150 µm. ICH, International Council of Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use; IHC, Immunohistochemistry; IND, Investigational new drug application; IXA, International Xenotransplantation Association; ISO, International Organization for Standardization; n.d., Not done; MVDL, University of Minnesota Veterinary Diagnostic Laboratory; NAHLN, National Animal Health Laboratory Network; NHP, Non-human primate; PAR-1, PERV-A receptor 1; PBMC, peripheral blood mononuclear cells; PBS, phosphate buffered saline; PCR, Polymerase chain reaction; qPCR, Quantitative PCR; Ph. Eur., European Pharmacopeia; PQA Plus®, Pork Quality Assurance Plus; PRNT, Plaque reduction neutralization test; RBC, Red blood cells; qRT-PCR, Quantitative reverse transcription PCR; SN, Serum neutralization; SOP, Standard operating procedures; SPF, Specific pathogen free; TEM, Transmission electron microscopy; USDA, United States Department of Agriculture; USP, United States Pharmacopeia; VI, Virus isolation; VN, Virus neutralization; WHO, World Health Organization

institutions indicate that an increasing number of islet recipients (approaching 50%) are able to maintain external insulin independence for 5 years.<sup>9</sup> Nonetheless, the availability of suitable human pancreases for either whole organ or isolated islet transplantation is entirely inadequate as evidenced by the recovery of only 1293 donor pancreases in the United States in 2015.<sup>10</sup>

The use of xenogeneic islets could provide a solution to the limited supply of human islets for transplantation. Porcine islets are particularly suitable as a source of insulin-producing cells because porcine insulin differs from human insulin by only a single amino acid and porcine insulin has been shown to provide reliable and safe glycemic control in human diabetic patients. Furthermore, only the transplantation of whole pancreas or intact islets of Langerhans can be expected to replicate normal and precise physiological glucose control, which is dependent on a variety of cell types and proteins from pancreatic islets. This enormously complex regulatory machinery is absent in all other approaches to the re-establishment of normoglycemia.

The use of animal-derived islet tissue, however, will necessitate either suppression of the patient's immune system or the physical isolation of the islets to prevent their rejection. We have previously described the ability of agarose-agarose encapsulated porcine islets to function in diabetic animal models.<sup>11-15</sup> These encapsulated islet macrobeads are at least partially immune isolated as an outer layer of dense agarose prohibits direct cell access to the xenogeneic islets. Although small immune mediators, such as cytokines, can still enter the macrobead, this encapsulation approach has been sufficiently robust to allow spontaneously diabetic BB rats to survive for more than 6 months without immunosuppression and without exogenous insulin administration.<sup>13</sup> Ongoing studies in non-immunosuppressed diabetic cynomolgus macaques also demonstrate porcine islet macrobead function for more than 6 months in 5 of 6 animals (unpublished data, see Checkpoint 4 below).

The clinical use of islets from animals also requires a well-thought-out strategy to assure the microbiological safety of the animal tissue. A strategy to provide an aseptic product and to reduce the probability of inadvertent transmission of adventitious viruses is critical to the successful implementation of this exciting therapy. Our approach takes advantage of the unique aspects of the islet macrobead, namely its long-term viability in culture and lack of patient immunosuppression, to limit the microbiological safety risk of porcine islets for transplantation. Essential to our approach is the establishment of four checkpoints that span the macrobead production and transplantation processes. The checkpoint approach is built upon an increasing level of microbiological screening from the donor herd up to the islet product, using multiple assay formats with the ability to screen for known and unknown pathogens. Using the checkpoint approach, we provide evidence to support the use of donor animals housed in high hygienic but not HEPA-filtered environments, not only for islet xenotransplantation but also other xeno-cell therapies. In this study, we present and document the effectiveness of our microbiological safety strategy to minimize the microbiological risks of agarose encapsulated porcine islets for the treatment of type 1 diabetes.

## 2 | MATERIALS AND METHODS

### 2.1 | Source animal facility

Donor pigs were Large White – Yorkshire × Landrace F1 hybrid animals obtained from well-characterized, non-genetically modified Choice Genetics (formerly Newsham) source animals. The Source Animal Facility is an independently owned herd located in rural Southwest Ohio. The breeding herd numbers approximately 2000 sows and utilizes a two-tiered quarantine testing system through a modern multisite production strategy. Three segregated facilities (nursery, gilt isolation, and farrowing and gestation) maintain data records and follow standard operating procedures (SOPs) for animal husbandry, vaccination, housing, cleaning and waste management, and biosecurity measures. Controlled transportation of animals, supplies and semen, feeding and watering, pest and rodent control, gravel barrier around facility perimeter, and caretaker qualifications including routine health assessments are all components of the comprehensive biosecurity program. Routine Quality Assurance site audits are used to verify compliance. Corn-based pig feed was produced exclusively with corn grown and ground onsite at the source animal facility. Gilts, gestation sows, and lactating sows were given the same base feed, consisting of corn with various supplements (eg, amino acids, vitamins, minerals, protein), with slight variations based on the breeding status of the animal. All ingredients used in the production of complete feed were certified to be free of any restricted use protein products as defined in the FDA Code of Federal Regulation Title 21 Part 589.2001 (21 CFR 589.2001) pertaining to cattle materials prohibited in animal feed. Complete medical histories are archived for all source animals. The facility holds Pork Quality Assurance Plus (PQA Plus<sup>®</sup>) certification, which is a producer-driven program to ensure U.S. pork products are of the highest quality and safe.<sup>16</sup> The PQA Plus program mandates Good Production Practices with an emphasis on biosecurity and practices to reduce the risk of introducing and spreading of infectious agents. All retired breeding sows not used as pancreas donors are sent to a local abattoir for pork products.

Source animals are routinely vaccinated for various agents (Table 1). Killed vaccines are used when available. Modified live virus vaccines are used with no evidence for reversion to virulence in back-passage studies (PARASAIL and ProSystem RCE) or when no USDA-licensed killed vaccines are available (Enterisol Ileitis). The modified live virus vaccine Ingelvac PRRS is the exception to the above [vaccine virus has been found in one study with non-vaccinated pigs in contact with vaccinated animals<sup>17</sup>] and is alternated quarterly with a killed PRRS vaccine.

The facility operates a Source Animal Health Screening Program to continuously assess the health status of the herd. Between January 2013 and December 2014, the screening program was composed of random antemortem monthly herd testing and a sentinel animal program that includes both antemortem and postmortem testing. Target agents, test methods, and sample types for the monthly and sentinel antemortem screening are shown in Table 2. The sentinel animal program consists exclusively of randomly selected breeding sows as they

**TABLE 1** Vaccine information and vaccination schedule

Vaccine	Target agent	Form	Manufacturer	Weaning	Post-weaning			Pre-farrowing	Post-breeding	Whole herd
					3 wk	6 wk	Gilts			
PARASAIL®	<i>Haemophilus parasuis</i>	Avirulent live	Newport Laboratories	X		X				
CircoFLEX®	Circovirus	Killed	Boehringer Ingelheim	X						
MycoFLEX®	<i>Mycoplasma hyopneumonia</i>	Killed	Boehringer Ingelheim	X						
Mycoshield®	<i>M. hyopneumonia</i>	Killed	Novartis		X					
Pneumostar® SIV	SIV H1N1 and H3N2	Killed	Novartis		X				X <sup>d</sup>	
Enterisol Ileitis	<i>Lawsonia intracellularis</i>	Attenuated	Boehringer Ingelheim			X				
Parvo Shield® L5E	Parvovirus, 5 strains of <i>Leptospira</i> and <i>Erysipelas</i>	Killed	Novartis			X		X		
Prefarrow Shield® 9 <sup>a</sup>	<i>E. coli</i> (K99, K88, 987P, F41), <i>C. perfringens</i> type C, <i>Bordetella bronchiseptica</i> , <i>Pastruella multocidatypus</i> A/D	Killed	Novartis				X			
ProSystem® RCE <sup>b</sup>	Rotavirus G serotypes 5 and 4 of Serogroup A, <i>C. perfringens</i> Type C, <i>E. coli</i> (K99, K88, 987P, F41)	Modified live	Merck				X			
Ingelvac® PRRS <sup>c</sup>	Porcine Reproductive and Respiratory Syndrome Virus	Modified live	Boehringer Ingelheim						X	
PRRS <sup>d</sup>	Porcine Reproductive and Respiratory Syndrome Virus	Killed	Newport						X	

<sup>a</sup>5 wk pre-farrowing.<sup>b</sup>2 wk pre-farrowing.<sup>c</sup>January and July.<sup>d</sup>April and October.

TABLE 2 Monthly herd and quarterly sentinel antemortem screening

		2013												
Target agent	Test method	Sample type	JAN	FEB	MAR	APR	MAY	JUN	JUL	AUG	SEPT	OCT	NOV	DEC
Monthly Herd	TGEV	Serum	0/49	0/50	0/50	0/55	0/50	0/50	0/56	1/50	0/51	0/50	0/50	0/50
	PRCV	Serum	44/49	49/50	47/50	50/55	46/50	50/50	55/56	34/50	25/51	29/50	10/50	16/50
	PRRSV*	Serum	32/49	26/50	48/50	47/55	30/50	27/50	29/56	36/50	32/51	26/50	48/50	45/50
Quarterly sentinel	<i>Brachyspira</i> sp.	Feces	n.d.	n.d.			n.d.			0/11			0/10	
	<i>Salmonella</i> spp.	Feces	2/10				0/10			0/11			0/10	
	Enteric viruses	Feces	0/10	0/10			10/10 <sup>a</sup>			2/8 <sup>b</sup>			10/10 <sup>c</sup>	
	Influenza A*	Nasal swab	0/10	0/10			4/10			0/11			0/10	
	PCMV	Buffy coat	0/10	0/10			0/10			0/11			0/10	
	PPV*	Serum	10/10 <sup>d</sup>	10/10 <sup>d</sup>			10/10 <sup>e</sup>			11/11 <sup>f</sup>			9/10 <sup>g</sup>	
	PRCV	Serum	10/10	10/10			10/10			10/11			2/10	
	PRRSV*	Serum	9/10	9/10			9/10			9/11			10/10	
	PRV	Serum	0/10	0/10			0/10			0/11			0/10	
	TGEV	Serum	0/10	0/10			0/10			0/11			0/10	
	BVDV-1.-2	Serum	0/10	0/10			0/10			0/11			0/10	
	EMCV	Serum	2/10 <sup>h</sup>	2/10 <sup>h</sup>			0/10			2/11 <sup>i</sup>			0/10	
	VSV-IN	Serum	0/10	0/10			0/10			0/11			0/10	
	VSV-NJ	Serum	0/10	0/10			0/10			0/11			0/10	
	EEEV	PRNT	0/10	0/10			1/10 <sup>j</sup>			2/11 <sup>j</sup>			0/10	
VEEV	PRNT	2/10 <sup>j</sup>	2/10 <sup>j</sup>			1/10 <sup>j</sup>			3/11 <sup>j</sup>			1/10 <sup>j</sup>		
WEEV	PRNT	2/10 <sup>j</sup>	2/10 <sup>j</sup>			1/10 <sup>j</sup>			3/11			1/10 <sup>j</sup>		
WNV	PRNT	0/10	0/10			0/10			0/11			0/10		

(Continues)



are the only animals housed long term (>2 years) at the facility. Blood, nasal swabs, and fecal samples are collected from sentinel animals for microbiological screening. Gilts selected from the nursery for breeding are first quarantined and qualified for introduction to the gestation facility. Once inside the breeding facility, breeding sows of all ages are candidates for the sentinel program. Trained boars used for heat-checking are not part of the sentinel program as they are not eligible for pancreas donation, although these animals are part of the routine herd health screening performed monthly.

## 2.2 | Postmortem microbiological screening of sentinel and donor animals

Following electrical stun and exsanguination at a USDA-regulated abattoir in compliance with all federal regulations concerning animal handling and welfare, numerous tissues including brain, heart, lung, liver, tonsil, lymph node, spleen, ileum, kidney, pancreas, bone marrow, PBMCs, feces, and serum were collected and archived as fixed and frozen aliquots at the time of pancreas procurement. Aliquots of isolated islets were also frozen and archived. Donor animal tissue samples were sent to the University of Minnesota, Veterinary Diagnostic Laboratory (MVDL) to screen for the bacterial and viral pathogens listed in Table 3. The MVDL is one of the three major swine diagnostic laboratories in the United States, and swine veterinarians use the MVDL's assays to monitor herd health and identify new outbreaks of infectious disease. The laboratory is a member of the National Animal Health Laboratory Network (NAHLN) of the USDA. A complete qualification report for all assays within the Herd Health Program, "Performance Characteristics of Diagnostic Assays Used to Screen Source Animals for Xenotransplantation," has been submitted to the FDA as part of an Investigational New Drug (IND) application for the use of agarose-agarose encapsulated porcine islets to treat patients with type 1 diabetes. The qualification report contains detailed information on all diagnostic assays used to screen source animals and includes assay protocols and data regarding sensitivity, specificity, and repeatability.

The MVDL assays are validated according to guidelines established by the American Association of Veterinary Laboratory Diagnosticians, and Standard Operating Procedures are available from the MVDL upon request (vdl@umn.edu). Detection of the different target agents included the following methods: Bacterial Culture, Transmission Electron Microscopy (TEM), Immunohistochemistry (IHC), conventional or quantitative Polymerase Chain Reaction (PCR or qPCR, respectively) and quantitative Reverse Transcription (qRT-PCR), Hemagglutination Inhibition (HI), Serum Neutralization (SN), Virus Isolation (VI), and Plaque Reduction Neutralization Test (PRNT). In addition, virus isolation assays were performed on the infection susceptible cells Madin-Darby canine kidney (MDCK) cells, porcine alveolar macrophages (PAM), primary porcine kidney cells (PPK), porcine kidney cells (PK-15), African green monkey kidney cells (MARC-145), and bovine turbinate cells (BT) to look for unknown porcine viruses. Each cell type was cultured in the presence of tissue homogenate (brain, ileum, kidney, lung, liver, mesenteric lymph node, spleen, and tonsil) from sentinel animals or donor animals and monitored for morphological changes indicative

of viral infection. The PRNT testing for EEEV, VEEV, WEEV, and WNV occurred at the National Veterinary Service Laboratory (Ames, IA, USA).

## 2.3 | Porcine islet isolation and encapsulation

Donor pancreases were procured from sows that were over 2 years of age with a history of multiple parities. Following electrical stunning and exsanguination of the donor, abdominal viscera were retrieved by a sterile-gowned technician and transferred to a sterile container for transport. The viscera were then placed in a custom built, HEPA-filtered, laminar flow work station for tissue retrieval. Surface monitoring was performed on the work surface prior to retrieval, and settle plates were placed inside the workstation during dissection to monitor for microbiological contamination. Intact pancreas was dissected from the viscera by a sterile-gowned technician and immediately transferred to cold HBSS (CellGro, Manassas, VA, USA). The tissue was then surface-rinsed by submersion in cold povidone-iodine 10% solution (VetOne, Boise, ID, USA), followed by two submersion rinses in cold HBSS. The pancreas was transported to the islet isolation laboratory in a sterile container submerged in cold HBSS. The container was then transferred to a biological safety cabinet where the pancreas was removed and a transport media sample collected for total bioburden testing (Avista Pharma Solutions, Inc., Agawam, MA, USA). Warm ischemia times (beginning of exsanguination to pancreas immersion in cold transport solution) ranged from 10 to 15 minutes. Cold ischemia times (pancreas immersion in cold transport solution until placement in digestion chamber) ranged from 42 to 57 minutes.

Pancreases were perfused with 7.5 Wunsch U/g collagenase (Collagenase MA; Vitacyte, Indianapolis, IN, USA), 12 000 Neutral Protease U/g pancreas (BP Protease; Vitacyte), and 2.5 mg/pancreas of Pulmozyme (Genentech, South San Francisco, CA, USA) solution prepared in Cold Storage Purification Stock Solution (CellGro). Islet counts were expressed as islet equivalents (IEQ), based on a standard islet size of 150  $\mu$ m, and 500 IEQ were encapsulated in agarose-agarose macrobeads as previously described.<sup>18</sup> Following quantification, islets were separated into 2000 IEQ aliquots before being resuspended in 0.5 mL of 0.8% (w/v) SeaKem Gold agarose (derived from *Gelidium* species of seaweed; Lonza Rockland, Inc., Rockland, MD, USA) prepared in Minimal Essential Medium plus 25 m/mol L HEPES buffer (Sigma-Aldrich, St. Louis, MO, USA). The islet-agarose suspension was expelled beneath the surface of sterile mineral oil (Sigma-Aldrich) to produce four 0.125-mL spherical beads of approximately 5-6 mm in diameter, each containing 500 IEQ. The 1st coat macrobeads were cultured at 37°C in a humidified atmosphere of 5% CO<sub>2</sub>. After 3-5 days, a second coat of 5% SeaKem Gold agarose was applied, giving each macrobead a final diameter of 8-9 mm. The determination of the average pore size of the agarose macrobeads is difficult at best, given the gelation mechanism of random polymer coils forming aggregated double helices, which results in pores of various diameters with ill-defined walls. For this reason, the macrobead has not been considered to provide absolute viral sequestration although the encapsulated islets are protected from direct immune cell contact. Agarase, the enzyme necessary to break down agarose, is abundant in ocean-dwelling

**TABLE 3** Sentinel and pancreas donor postmortem screening

Target agent	Test method(s)	Sample type	2013		2014	
			Sentinels	Pancreas donors	Sentinels	Pancreas donors
Aerobic	Culture	Lung	0/3	0/6	n.d.	0/2
<i>Brachyspira</i> sp.	Culture	Feces	0/3	0/7	n.d.	0/1
<i>Salmonella</i> spp.	Culture	Feces, mesenteric lymph node, spleen, tissue pool <sup>a</sup>	0/3	0/7	n.d.	0/2
<i>Leptospira</i> *	IHC	Kidney (formalin-fixed)	0/3	0/7	0/3	0/4
<i>L. intracellularis</i> *	IHC	Ileum (formalin-fixed)	0/3	0/7	0/2	0/4
TGEV	IHC	Ileum (formalin-fixed)	0/2	0/7	0/3	0/3
Enteric viruses	TEM	Feces	2/3 <sup>b</sup>	1/7 <sup>c</sup>	0/3	1/3 <sup>d</sup>
Influenza A*	qRT-PCR	Lung	0/3	0/7	0/3	0/4
PCMV	qPCR	Buffy coat	0/3	0/7	0/3	0/4
BVDV-1, -2	qRT-PCR	Tissue homogenate <sup>e</sup>	0/3	0/6	0/3	0/4
BVDV-1, -2	VI (culture: BT)	Tissue homogenate <sup>e</sup>	0/3	0/7	0/3	0/4
EMCV	VI (culture: BHK)	Serum, heart	0/3	0/7	0/3	0/4
PHoV	qPCR	Pancreas	n.d.	2/7	0/1	1/1
PHoV	qPCR	Mesenteric lymphnode	1/3	2/7	1/3	3/3
PERV-A	qRT-PCR	Tissue homogenate <sup>e</sup>	3/3	7/7	3/3	4/4
PERV-B	qRT-PCR	Tissue homogenate <sup>e</sup>	3/3	7/7	3/3	4/4
PERV-C	qRT-PCR	Tissue homogenate <sup>e</sup>	2/3	5/7	3/3	4/4
PRRSV	qRT-PCR	Serum, tissue homogenate <sup>e</sup>	0/3	0/7	0/3	0/4
PRRSV*	ELISA	Serum	2/3	7/7	3/3	3/4
PRCV	ELISA	Serum	2/3	6/7	2/3	2/4
PRV	ELISA	Serum	0/3	0/7	0/3	0/4
TGEV	ELISA	Serum	0/3	0/7	1/3	0/3
VSV-IN	SN	Serum	0/3	0/7	0/3	0/4
VSV-NJ	SN	Serum	0/3	0/7	0/3	0/4
EEEV <sup>f</sup>	PRNT	Serum	1/3 <sup>g</sup>	0/7	1/3 <sup>g</sup>	0/4
VEEV <sup>f</sup>	PRNT	Serum	0/3	1/7 <sup>g</sup>	1/3 <sup>g</sup>	0/4
WEEV <sup>f</sup>	PRNT	Serum	0/3	2/7 <sup>g</sup>	0/3	0/4
WNV <sup>f</sup>	PRNT	Serum	0/3	0/7	0/3	0/4
PPV*	HI	Serum	3/3 <sup>h</sup>	6/6 <sup>i</sup>	3/3 <sup>j</sup>	4/4 <sup>k</sup>
PHEV	HI	Serum	2/2 <sup>l</sup>	3/3 <sup>m</sup>	n.d.	n.d.
Porcine viruses	Culture: MDCK	Tissue homogenate <sup>n</sup>	0/3	0/7	0/3	0/4
Porcine viruses	Culture: PAM	Tissue homogenate <sup>n</sup>	0/3	0/7	0/3	0/4
Porcine viruses	Culture: PPK	Tissue homogenate <sup>n</sup>	0/3	0/7	0/3	0/4
Porcine viruses	Culture: PK-15	Tissue homogenate <sup>n</sup>	0/3	0/7	0/3	0/4
Porcine viruses	Culture: MARC-145	Tissue homogenate <sup>n</sup>	0/3	0/7	0/3	0/4
Porcine viruses	Culture: BT	Tissue homogenate <sup>n</sup>	0/3	0/7	0/3	0/4

\*Vaccination, [n.d. (not done)], <sup>a</sup>colon, ileum, liver, spleen, lung, and mesenteric lymph node, <sup>b</sup>coronavirus (n = 1), caudovirales (n = 1), <sup>c</sup>small round viral particle, <sup>d</sup>dilution factor (df): 10, <sup>e</sup>kidney, lung, mesenteric lymph node, tonsil, and spleen, <sup>f</sup>Testing performed at National Veterinary Service Laboratory, <sup>g</sup>bacteriophage, <sup>h</sup>df: 512 (n = 1), 1024 (n = 2), <sup>i</sup>df:1024 (n = 2), 2048 (n = 2), 8192 (n = 2), ≥8192 (n = 1), <sup>j</sup>df: 1024 (n = 1), 2048 (n = 1), 4056 (n = 1), <sup>k</sup>df: 512 (n = 2), 2048 (n = 2), <sup>l</sup>df: 20 (n = 1), 40 (n = 1), <sup>m</sup>df: 40 (n = 1), 320 (n = 1), 640 (n = 1), <sup>n</sup>heart, intestine, liver, pancreas, kidney, lung, mesenteric lymph node, tonsil, and spleen.

bacteria but has not been found in mammals.<sup>19</sup> The macrobeads, in the absence of trauma, remain intact indefinitely following implantation in the abdominal cavity.

Macrobeads were then cultured at 37°C in a humidified atmosphere of 5% CO<sub>2</sub> until collection for microbiological screening or implantation. Culture medium (RPMI 1640; Life Technologies, Grand Island,



NY, USA; pre-screened for endotoxin <0.03 EU/mL and for sterility) containing 11 mmol/L glucose, 2.5% heat-inactivated porcine serum (Biologos, Montgomery, IL, USA; pre-screened for sterility, and the absence of mycoplasma and adventitious viruses per 9CFRs and endotoxin <50 EU/mL) and 1% antibiotic/anti-mycotic (Life Technologies) was changed weekly and 24-hour post-change media samples were taken for porcine insulin ELISA assays (Mercoxia, Uppsala, Sweden). Islet isolation and encapsulation procedures were performed in Class II biosafety cabinets within an ISO Class 5 (at rest) and ISO Class 7 (active processing) laboratory by sterile-gowned technicians. Routine environmental monitoring included particle counts, viable particle counts, settling plates, and surface monitoring and was performed for every islet isolation procedure. Macrob beads from a given donor were assigned a unique islet isolation number and cultured separately from islet macro beads produced from other donor animals.

## 2.4 | Viral screening of porcine islet macro beads

All viral screening of islet macro beads was performed at SGS Vitrology (Glasgow, UK). This group has extensive experience (approximately 15 years) with the testing of both the islet macro beads and cancer macro beads (see Discussion) and has been essential in the development of the viral screening programs for each product. Representative macro beads were sealed in sterile screw top tubes, frozen, and shipped on dry ice to SGS Vitrology for screening using both molecular and in vitro co-culture assays. All shipments were received and released by the Office of the Scottish Executive Rural Directorate following appropriate content and importation license verification. Aliquots of islet macro beads, rather than isolated free islets, were selected for screening to insure any potential viral activation due to the stress of the encapsulation procedure was accounted for. Similar aliquots were also frozen and archived for possible future investigative use. For PCR assays, DNA was extracted from 6 macro beads pooled from 3 individual donors (two macro beads per donor) after homogenization in Buffer ATL (Qiagen, Valencia, CA, USA), lysis, and adsorption to a silica membrane. A Qiagen RNeasy kit was used for RNA extraction according to the manufacturer's instructions. The viral RNA and DNA extraction methods and the TaqMan<sup>®</sup> real-time PCR assays were validated to ICH Q2 guidelines. All testing was carried out with validated assays, and all PCR tests were performed in compliance with, and as described by Ph. Eur. 2.6.21<sup>20</sup> and USP <1127><sup>21</sup> (Figure 1). Each reaction contained 15 000–30 000 islet cell equivalents (qPCR) or 13 000–26 000 islet cell equivalents (qRT-PCR).

In vitro assays for the detection of adventitious virus contaminants were performed as follows. Six islet macro beads per donor sample, in culture medium, were used to generate each test sample. The test material was thawed, supernatant retained, and the macro beads then ground in a sterile tissue grinder and taken through two cycles of freezing and thawing. The ground beads were then mixed with the culture supernatant and the pooled material filtered through a 100 µm filter to remove large debris and clarified by centrifugation. The clarified supernatant material was then used as the lysate sample for inoculation. Four indicator cell lines consisting of human lung fetal fibroblast cells

(MRC-5), baby hamster kidney cells (BHK-21), swine testis cells (ST), and African green monkey kidney cells (MA104) were inoculated with each test sample, incubated at 36°C (±1°C), and monitored for a period of at least 28 days for development of virus-induced cytopathic effect (cpe). Negative controls included monitoring the health of the indicator cell lines in tissue culture medium only throughout the in vitro assay. Cytopathic (cpe) virus positive controls were inoculated on the same day as the test samples and were included for each indicator cell line: MRC-5 cells inoculated with Herpes simplex virus type 1; BHK-21 cells inoculated with EMCV; ST cells inoculated with PPV; MA104 cells inoculated with Reo-3. The positive control viruses were inoculated at 100 TCID<sub>50</sub> units/vessel. At the end of the assay, negative controls (tissue culture medium only) and a positive control (Influenza A virus) for the hemadsorption (HA) assay were included to qualify each of the red blood cell preparations, using a pool of guinea pig, chicken, and human type O red blood cells, at 2–8°C. These controls were inoculated onto separate batches of indicator cells nearer the end of the assay.

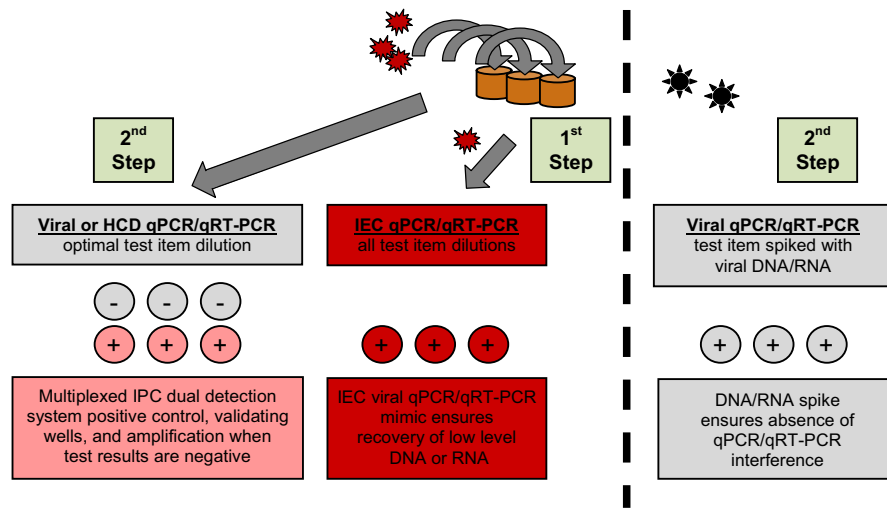
Transmission electron microscopy (TEM) was performed using RPMI/2.5% Glutaraldehyde fixed islet cells in macro beads. The cells were post-fixed in osmium tetroxide, stained *en bloc* in uranyl acetate, dehydrated in an ethanol series and propylene oxide before being infiltrated to Araldite resin and polymerized. Semi-thin sections (~1 µm) were cut and mounted on glass slides, stained with toluidine blue, and examined by light microscopy for general quality of fixation, gross cell morphology, and for the presence of mitotic cells and dead or dying cells in the culture. Ultrathin sections (0.1 µm) were cut, mounted on electron microscope grids, and stained with uranyl acetate and lead citrate solutions. Minimums of 200 median cell profiles were examined for the presence of viruses, virus-like particles, and other extraneous agents. The ultra structural morphology of the cells was also recorded.

## 2.5 | Sterility, mycoplasma, and endotoxin screening of islet macro beads

Prior to islet macro bead implantation, representative samples of macro beads were sent for sterility testing per USP <71><sup>22</sup> and Ph. Eur. 2.6.1<sup>23</sup> via membrane filtration and additionally assessed for the presence of Mycoplasma using direct and indicator cell culture methods (USP <63><sup>24</sup> and Ph. Eur. 2.6.7<sup>25</sup> Mycoplasma Tests; Avista Pharma Solutions, Inc. Agawam, MA, USA). Macro beads were also screened for the presence of bacterial endotoxins per USP <85><sup>26</sup> and Ph. Eur. 2.6.14.<sup>27</sup> Microbiological testing (sterility, endotoxin, and mycoplasma) was repeated 2–4 weeks prior to implant, as well as 24-hour prior to implant.

## 2.6 | Macro bead implant and viral screening of diabetic non-human primates

Six non-immunosuppressed streptozotocin-induced diabetic male cynomolgus macaques of Mauritian or Asian origin with a median age of 6.7 years (range 4.0 to 8.9 years) and median body weight of 7.6 kg (range 5.5 to 8.4 kg) each received 198–315 (mean 246) porcine islet macro beads implanted intraperitoneally (day 0), corresponding to a



**FIGURE 1** Viral Screening of Porcine Islets by qPCR / qRT-PCR. Overview of the qPCR/qRT-PCR (as appropriate) testing strategy used in the viral screening of porcine islet macrobeads (Checkpoint 3). 1st Step: each test sample is spiked with an internal extraction control (IEC; plant RNA or DNA) at the detection limit, mimicking the target nucleic acid. This process allows the level of nucleic acid recovery in the presence of the test sample to be assessed, which ensures that low levels of RNA or DNA can be recovered. 2nd Step: Test samples contain viral target primers/probes, multiplexed with internal positive control (IPC) reagents during amplification. In a separate assay, test samples are spiked with target nucleic acid (viral RNA or DNA) at the detection limit. This process ensures any negative results are not caused by sample matrix interference of the qPCR assay. This method of viral screening complies with the specific testing approach using PCR detection as described in Ph. Eur. 2.6.21, USP <1237>, and FDA Vaccine Guidance to Industry (2010)

mean of 123 000 islet equivalents per animal. All six animals received a second dose of 152-362 (mean 200) islet macrobeads between 138 and 372 days from the first transplant corresponding to a mean of 100 000 islet equivalents per animal. Blood samples for PBMC isolation were collected prior to and post-transplant at scheduled intervals for subsequent PCR analysis to determine potential porcine viral transmission including PCV, PLHV, PRRSV, PCMV, and PERV-A, PERV-B, and PERV-C infection. Time points evaluated were 1 month ( $\pm 7$  days) and 6 months ( $\pm 15$  days) post-transplant. Additionally, samples from 1 year ( $\pm 60$  days), 2 years ( $\pm 60$  days), and 3 years ( $\pm 60$  days) post-transplant were evaluated in a subset of recipients who reached these time points during extended follow-up. All procedures in animals were approved by the University of Minnesota Institutional Animal Care and Use Committee, conducted in compliance with the Animal Welfare Act, and adhered to principles stated in the Guide for Care and Use of Laboratory Animals.

### 3 | RESULTS

#### 3.1 | Multiple checkpoints for microbiological safety

Multiple checkpoints during the process of porcine islet isolation, encapsulation, culture, and transplantation were implemented to assess the microbiological safety of islet macrobeads (Figure 2).

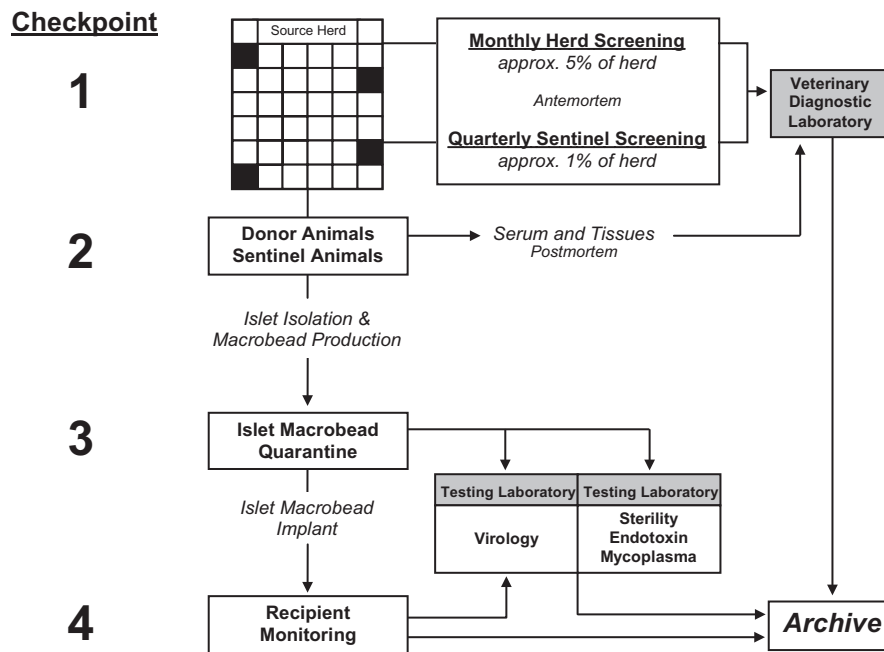
#### 3.2 | Checkpoint 1: High health status animals

The health status of all animals at the Source Animal Facility is maintained at a high level and continuously evaluated through the use of

monthly health checks from randomly selected animals and the utilization of a Sentinel Animal Program in which selected animals located throughout the facility act as environmental monitors through extensive and repeated screenings. Monthly screening of randomly selected animals during the years of 2013 and 2014 for the presence of antibodies to PRRSV confirmed the efficacy of the vaccination program [826/1207 PRRSV antibody-positive (68%)] given the absence of PRRSV RNA in sentinel and donor animal screening (Table 2). Antibodies were also routinely detected for PRCV (52%), demonstrating the widespread prevalence of this virus. Because the donor animal facility is negative for the presence of TGEV, screening for the introduction of this virus was used to continuously document the biosecurity of the Source Animal Facility: the one positive finding from 1207 samples was considered a false positive given the absence of any further positive findings.

Quarterly antemortem screening results of sentinel animals also demonstrate the efficacy of the vaccination program and overall herd health as evidenced by positive findings of antibodies to PPV and PRRSV in 150 of 162 samples (92%) that are part of the vaccination program, and no evidence of Influenza A virus as monitored by the presence of the RNA, which is also part of the vaccination program (0 of 81 nasal swab samples; Table 2). Additional results from sentinel animals included 42 positive identifications from 78 fecal samples (54%) for bacteriophage/*Picornaviridae*/*Caudovirales* in feces using electron microscopy. Antibodies to PRCV were found in 48 of 81 serum samples (59%). A total of 29 results were considered suspect for the Equine encephalomyelitis viruses (Venezuelan, Western, and Eastern) owing to virus growth variation and interpretation of low dilution factor results with these serology-based assays. Seven positive results for the serology-based EMCV assay and four findings of *Salmonella* spp. in feces were also reported.

## Porcine Islet Macrobead Biosafety Program



**FIGURE 2** Porcine Islet Macrobead Biosafety Program. Various checkpoints are established during the lifecycle of porcine islet macrobeads to support microbiological safety. Checkpoint 1 encompasses a comprehensive set of biosecurity protocols for the management of the Source Animal Facility and includes monthly viral screening of randomly selected animals as well as quarterly screening of Sentinel Animals. In Checkpoint 2, all pancreas donor animals and culled Sentinel Animals undergo thorough necropsies with numerous tissues screened and archived. In Checkpoint 3, islet macrobeads are quarantined in tissue culture, while representative samples of each production lot are screened for the presence of 32 known viruses using validated molecular assays. Co-culture assays and electron microscopy screening of islet macrobeads are also used to screen for unknown viruses in Checkpoint 3. Samples from recipients of porcine islet macrobeads can be similarly screened under Checkpoint 4

### 3.3 | Checkpoint 2: Postmortem screening of sentinel animals and pancreas donor animals

Numerous tissues were collected from all sentinel and donor animals at sacrifice for viral screening at the MVDL. Postmortem viral screening of six sentinel animals and 11 pancreas donor animals during 2013 and 2014 yielded 34 positive results from 301 samples (11%), excluding the viruses that are part of the vaccination program, the three PERV subtypes, and the samples screened with co-culture assays (Table 3). In 2013, the presence of enteric viruses was detected in 2 of 3 sentinel animals (Coronaviridae,  $n = 1$ ; Caudovirales,  $n = 1$ ), and a small, round viral particle was observed but not identified in the feces of 1 of 7 pancreatic donor animals. In 2014, a bacteriophage was identified in 1 of 2 pancreas donor animals tested, while no positive findings were observed in the feces of the three sentinel animals tested. Screening for PHEV antibodies yielded positive findings in 2 of 2 sentinel animals and 3 of 3 pancreas donor animals in 2013. Further screening was discontinued in source animals in 2014 as this virus has been shown to be ubiquitous in pig herds and has no public health significance given that the pig is the only known host.<sup>28</sup> Six positive results observed for the three Equine encephalomyelitis viruses are considered suspect-positive results given the low dilution factor

reported ( $df = 10$ ). A single positive result was reported for antibodies to TGEV in the postmortem serum sample of a sentinel animal in 2014. The antemortem serum from this same animal was consistently negative, and immunohistochemistry staining of the ileum was also negative. The presence of PHoV sequences by PCR analysis was detected in both sentinel and donor animals (3 of 9 pancreases and 7 of 16 lymph node samples). In subsequent assessment of isolated porcine islets, there was no evidence of this virus. No evidence of viral transmission was observed in six different cell lines from four species using tissue homogenate samples from each of the sentinel and donor animals.

### 3.4 | Checkpoint 3: Quarantine of islet macrobeads for microbial and viral screening

Given evidence of satisfactory insulin production by cultured islet macrobeads ( $\geq 75$  mU/macrobead/24-hour), the macrobeads were sent for viral screening to SGS Vitrology prior to use in pre-clinical animal studies. All islet macrobeads were negative for viruses screened by PCR ( $n = 32$  viruses) and by co-culture assays using four different susceptible cell lines ( $n = 12$  pancreas donor animals; Table 4). Transmission scanning electron microscopy was incorporated as an

**TABLE 4** Porcine islet macrobead screening

Porcine viral screening by qPCR/qRT-PCR								
Lot (isolation dates):	062812-092012-101012		101612-112912-121312		010913-012413-041113		121213-011614-021314	
Target agent	Sensitivity <sup>a</sup>	Result	Sensitivity <sup>a</sup>	Result	Sensitivity <sup>a</sup>	Result	Sensitivity <sup>a</sup>	Result
BDV	1000	(-)	100	(-)	100	(-)	100	(-)
BVDV-1	1000	(-)	100	(-)	100	(-)	1000	(-)
BVDV-2	1000	(-)	1000	(-)	1000	(-)	1000	(-)
PEV	1000	(-)	100	(-)	100	(-)	100	(-)
PAdV	10 000	(-)	10 000	(-)	10 000	(-)	10 000	(-)
PCV	50	(-)	50	(-)	50	(-)	50	(-)
PCMV	10	(-)	10	(-)	10	(-)	10	(-)
PHEV	25	(-)	25	(-)	25	(-)	25	(-)
PLHV-1, -2, -3	100	(-)	100	(-)	100	(-)	100	(-)
PPV	10	(-)	10	(-)	10	(-)	10	(-)
PRotA	100	(-)	100	(-)	100	(-)	100	(-)
PRotC	10 000	(-)	10 000	(-)	10 000	(-)	10 000	(-)
PRV	100	(-)	100	(-)	100	(-)	100	(-)
PTV	1000	(-)	1000	(-)	1000	(-)	1000	(-)
Rabies	100	(-)	100	(-)	100	(-)	100	(-)
REO-1, -2, -3	1000	(-)	1000	(-)	1000	(-)	1000	(-)
SEOV	10	(-)	10	(-)	10	(-)	10	(-)
SNV	1000	(-)	1000	(-)	1000	(-)	1000	(-)
EMCV	1000	(-)	1000	(-)	1000	(-)	1000	(-)
HEV	1000	(-)	1000	(-)	1000	(-)	1000	(-)
SVDV	100	(-)	100	(-)	100	(-)	100	(-)
SWPOX	10	(-)	10	(-)	10	(-)	10	(-)
TTV	100	(-)	100	(-)	100	(-)	100	(-)
TGEV	10	(-)	10	(-)	10	(-)	10	(-)
EEEV	100	(-)	100	(-)	100	(-)	100	(-)
VEEV	100	(-)	100	(-)	100	(-)	100	(-)
WEEV	100	(-)	100	(-)	100	(-)	100	(-)
WNV	100	(-)	100	(-)	100	(-)	100	(-)

(Continues)

TABLE 4 (Continued)

28-day in vitro co-culture assay for detection of viral contaminants																																						
Isolation Date:	062812			092012			101012			101612			112912			121312			010913			012413			041113			121213			011614			021314				
Cell Line	cpe	HA	HA	cpe	HA	HA	cpe	HA	HA	cpe	HA	HA	cpe	HA	HA	cpe	HA	HA	cpe	HA	HA	cpe	HA	HA	cpe	HA	HA	cpe	HA	HA	cpe	HA	HA					
MRC-5	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)				
BHK-21	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)			
ST	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)		
MA104	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)		
Microbiological screening of porcine islet macrobeads																																						
Isolation Date:	062812			092012			101012			101612			112912			121312			010913			012413			041113			121213			011614			021314				
Test	Sterility	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)		
Endotoxin <sup>b</sup>	0.006	<0.005	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	
Mycoplasma	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)
TEM	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.

Cpe, cytopathic effect; HA, hemadsorption.

<sup>a</sup>sensitivity = limit of detection (gene copies per PCR).

<sup>b</sup>EU/macrobead, transmission electron microscopy (TEM), not done (n.d.).

additional viral screening tool in 2013 and no viruses, virus-like particles or extraneous agents including mycoplasma, yeasts, fungi, or bacteria were found in the 6 pancreas donors comprising the two macrobead Lots produced in 2013 and 2014 (Table 4 and Figure 3). Conditioned media samples taken at 4 weeks post-islet encapsulation were negative for bacterial growth and for the presence of endotoxin and mycoplasma (Table 4).

### 3.5 | Checkpoint 4: Recipient screening for transmission of infectious agents

Although not a source material or product release screen, recipient monitoring post-macrobead implantation provides valuable data that could be used to not only treat individual patients but also as a check for the efficacy and possible modification of the pre-implantation checkpoints and is an important component of a thorough safety program. As an example of islet macrobead recipient monitoring, 6 diabetic cynomolgus macaques received porcine islet macrobead implantations and were routinely monitored for graft function and evidence of viral transmission and/or infection. Five of 6 animals implanted have shown a measurable clinical benefit in terms of reduced exogenous insulin requirements (an approximately 50% reduction as designed to model a proposed Phase 1 IND trial) with improved and/or stable blood glucose readings and HbA1c levels for up to 4 years. No significant adverse events have been observed in these animals to date. Body weight has remained stable or increased in all animals in line with the overall health of these animals. Screening for selected viruses was performed at the MVDL and was based on the presence of the virus in the source herd (PCV [Type 2], PERV-A, PERV-B, PERV-C), viruses with uncertain herd status (PRRSV) or viruses of concern given similarity to infectious human viruses or activation in pig to immunosuppressed primate xenotransplantation (PLHV-1, 2, and 3 and PCMV, respectively). Isolated peripheral blood mononuclear cells from all time points were negative for PCV (Type 2), PLHV-1, 2, and 3, PRRSV, PCMV, PERV-A, PERV-B, and PERV-C by PCR analysis (Table 5). Moreover, there has been no evidence of viral infection as observed and documented in the health status of the animals.

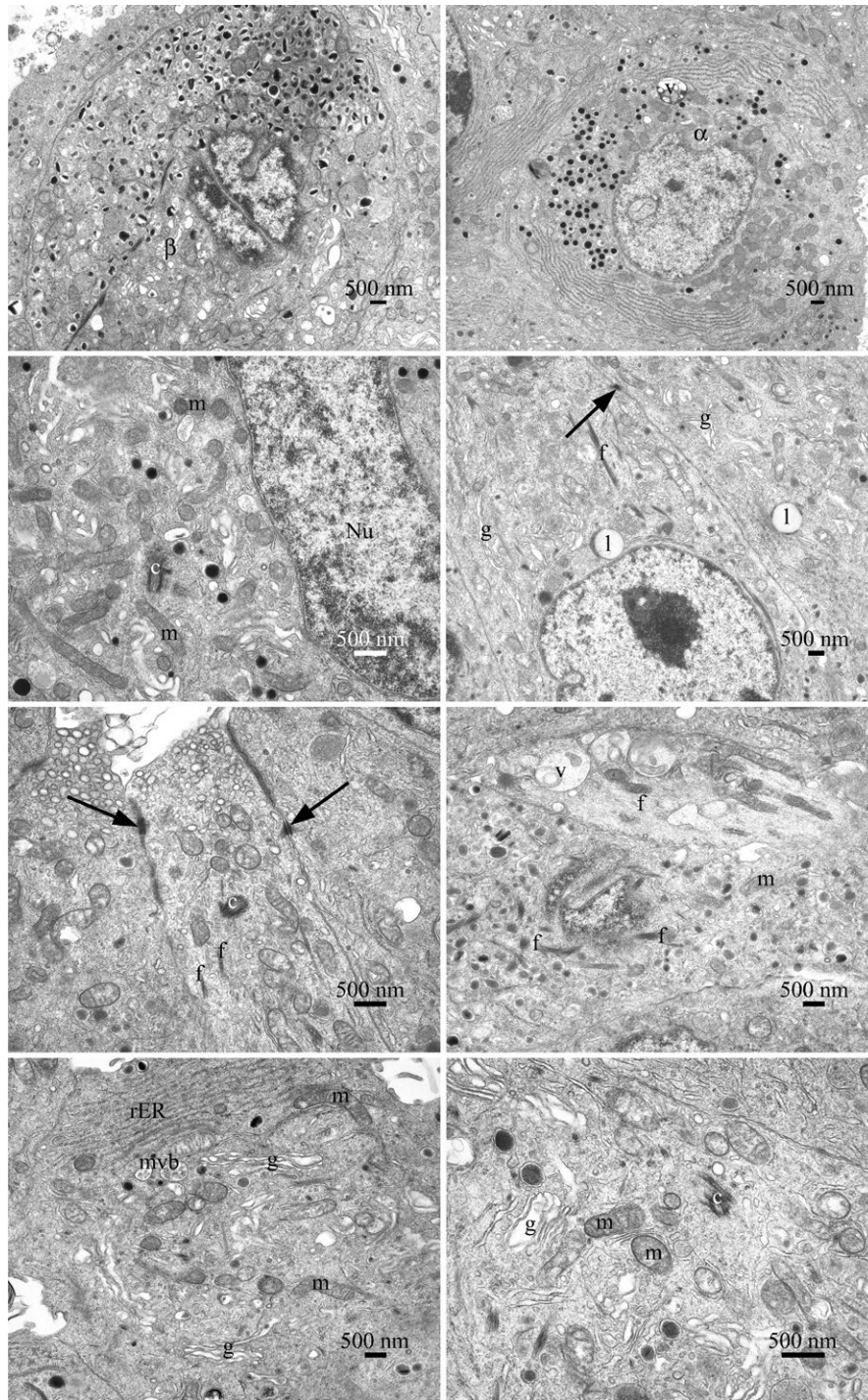
## 4 | DISCUSSION

The four-checkpoint microbiological safety program outlined utilizes four separate assessments for the microbiological safety of agarose encapsulated porcine islets. These occur sequentially from the source animal herd and individual donor animals to the intended transplant product and finally the recipient following implantation. The donor herd, designated sentinel animals, pancreas donor animals, and islet macrobeads are extensively screened to exclude latent and adventitious contaminants. The encapsulation method supports islet viability over extended culture periods, which permits ample time for the conduct of numerous screening assays for the detection of specific viruses, electron microscopic detection of viral particles and/or cell damage, and the lengthy *in vitro* culture screens for both known and

unknown viruses. This is a significant advance over the indirect testing of animals raised under gnotobiotic conditions and allows for screening at multiple points throughout the islet macrobead manufacturing process. Finally, samples from patients implanted with porcine islet macrobeads can be screened for the evidence of xenozoonosis using validated assays similar to those we have employed for sentinel and donor animals and for macrobead testing as part of a post-implant surveillance program.

This integrated safety program has been developed with the help of numerous scientists, clinicians, and experts in veterinary care, toxicology, quality assurance, veterinary diagnostics, islet isolation, clinical medicine, microbiology, and virology. The program has evolved from the adventitious agent screening assessments used for vaccines and biotechnology products described in the USP, Ph. Eur., and in the ICH guidelines. Certain adaptations have been made due to the nature of the samples and type of product. The ability of this program to ensure that the health status of donor animals is high and that islet macrobeads are consistently negative for bacterial or viral contamination is now firmly established. The safety program is open to continual review and refinement in consultation with relevant experts throughout the world. For example, the use of transmission electron microscopy (TEM) to provide an additional screen of islet macrobeads for known and unknown viruses and for other extraneous agents has been recently implemented as it is routinely applied in vaccine production, even though TEM has a considerably lower sensitivity compared to PCR. Any positive TEM findings will be investigated to identify known agents using immunohistochemistry or molecular techniques. The involvement of various specialists extends beyond the development of the microbiological safety program and encourages continual program improvement. Regular meetings, for example, could prompt the addition of new viruses of concern to be added to the screening panels or new/optimized procedures from animal husbandry to clinical care could be implemented under specialist guidance.

In the United States, the Food and Drug Administration (FDA) has regulatory responsibility for somatic cell therapy that includes xenogeneic cells and therefore porcine islets.<sup>29</sup> The FDA Center for Biologics, Evaluation and Research (CBER) regulates biological products including cell therapies and released a final Guidance for Industry regarding xenotransplantation products in 2003.<sup>30</sup> The European Medicines Agency (EMA) oversees Advanced Therapy Medicinal Products (ATMPs) including cell transplantation in Europe.<sup>31-33</sup> The World Health Organization<sup>34,35</sup> as well as the International Conference on Harmonisation<sup>36-39</sup> have also issued xenotransplantation guidance. The International Xenotransplantation Association (IXA) recently published a first update to the original consensus statements on the use of porcine islets for clinical trials.<sup>40-47</sup> The focus of all these guidelines, including the IXA statements, is on the safety of xenotransplantation.<sup>30,33</sup> An invited review of US xenogeneic regulations was published by FDA staff in 2010 and provides additional details on FDA guidelines.<sup>48</sup> Schuurman has more recently published a thorough overview of worldwide xenogeneic regulations with an emphasis on the European requirements.<sup>49</sup>



**FIGURE 3** Transmission electron microscopy of islet cell profiles, typical of those present in the sample of cells examined. The islet cells were generally adherent with intercellular connections, cell junctions, and desmosomes observed. The cell surface appearances varied, smooth surfaces, blebs, and cell processes were observed. Various cell types were present, including  $\alpha$  and  $\beta$  cells. No mitotic cells were observed by light or electron microscopy. No significant numbers of dead or dying cells were present in the population of cells examined. Nuclear profiles varied in size. In some cells, more than one nuclear profile was visible. They were often indented and invaginated or contained lacunae of cytoplasmic material. Nucleoli were prominent and sometimes more than one was observed. Heterochromatin was abundant, clumped, and found on the inner nuclear membrane and sporadically throughout the nucleus. The cells had a normal range of organelles. The mitochondria were numerous and varied in size and shape. The presence of cristae was noted although no matrix granules were seen. The Golgi body, when observed, was prominent and exhibited stacked cisternae. The rough-surfaced endoplasmic reticulum was extensive and occurred in varied lengths. Lipid bodies, peroxisomes, and multivesicular bodies were present. Free ribosomes, polysomes, and fibrils were found throughout the cytoplasm. Vacuoles, with electron dense and flocculent material, were observed. Coated, un-coated, and secretory vesicles, of varying types, were seen. Centrioles and microtubules were present. The cell structure is consistent with the morphology expected of secretory cells. No viruses, virus-like particles or extraneous agents, including mycoplasmas, yeasts, fungi, or bacteria, were found. Abbreviations: f, fibrils; mitochondria (m); desmosome (arrow); centriole (c); nucleus (Nu); Golgi bodies (g), lipid bodies (l), vacuoles (v), multivesicular body (mvb), and rough-surfaced endoplasmic reticulum (rER)

**TABLE 5** PCR screening of implanted non-human primates

Target agent	PCV, PLHV, PRRSV, PCMV, PERV					
	Time (Post-transplant)					
Animal ID	1 month	6 months	1 year	2 years	3 years	4 years
08FP6	(-)	(-)	(-)	(-)	(-)	(-)
08KP5	(-)	(-)	(-)	(-)	(-)	(-)
12JP3	(-)	(-)	(-)	(-)		
12JP2	(-)	(-)	(-)	(-)		
08FP10	(-)	(-)	(-)			
08FP17	(-)	(-)	(-)			

Time points evaluated were: 1 month ( $\pm 7$  days) and 6 months ( $\pm 15$  days) post-transplant. Additionally, samples from 1 year ( $\pm 60$  days), 2 years ( $\pm 60$  days), 3 years ( $\pm 60$  days) and 4 years ( $\pm 60$  days) post-transplant were evaluated in a subset of recipients who reached these time points during extended follow-up.

Our microbiological safety program is in-line with the issues for consideration in the FDA xenotransplantation guideline, as well as the IXA statements. In regard to donor animals, it is important to note that the guidelines do not state that source animals must be raised in HEPA-filtered environments. This is significant because, in addition to the difficulty of obtaining and maintaining pathogen-free swine,<sup>50–52</sup> there is no guarantee that the pancreas from individual animals will be suitable for islet isolation. In fact, it is becoming increasingly clear that only about 25% of porcine pancreases yield a sufficient number of islets for clinical use.<sup>18,53–55</sup> Given these limitations, our approach is to raise source animals in high hygienic environments, biopsy individual pancreases for islet isolation suitability and perform extensive microbiological screening of all selected donor animal tissues and the islet macrobeads themselves.

Our methodology, however, employs an alternative approach to the FDA-recommended 21-day quarantine period for individual donor animals that deserves some discussion. A minimum 7-day quarantine period for animals used in the processing of biological products is also stated in 21 CFR part 600.<sup>56</sup> The rationale for the quarantine period is to provide time for an acute infection to become clinically apparent.<sup>57</sup> During a quarantine period, cohort animals would be housed in a HEPA-filtered environment with strict husbandry practices designed to eliminate the introduction of adventitious agents, similar to a laboratory specific pathogen-free (SPF) animal facility. Such animals would be monitored for the development of disease symptoms and undergo blood, fecal, and nasal swab sampling for microbiological status assessment. The development of disease symptoms or unsatisfactory diagnostic results in any animal would disqualify all animals in a given cohort from pancreas donation. This donor qualification process is most appropriate when there is not sufficient time to perform thorough screening of the islets to be transplanted, for example, when free islets are to be transplanted within a few days of isolation. The islets encapsulated in the agarose macrobeads, in contrast, survive for more than 1 year during *in vitro* culture<sup>13</sup> allowing ample time to conduct the numerous and lengthy assays designed to detect both known and unknown microbiological agents. Assays that screen the islet product directly are more likely to detect a pathological agent

than the assessment of the clinical condition of the donor which may not present disease symptoms (eg, PPV) or the detection of a latent virus (eg, PLHV) that might be readily observed in particular organs but not as reliably in peripheral blood cells.<sup>58</sup> Thus, it is the islet macrobeads themselves, that is, the tissue to be implanted, that undergoes quarantine and it is the ability to thoroughly screen the islets using a wide variety of specific and non-specific assays that obviates the need to raise or quarantine donor animals in HEPA-filtered environments. As discussed by Schuurman in the first IXA consensus statement on source animals, "...it can be suggested that with longer time periods of islet cell culture the relevance of health status of the source pig becomes less, and that the focus of DPF status is more on the cultured cells."<sup>59</sup> This ideology is again reflected in the first update of the IXA consensus statements in relation to encapsulated islets allowing the time necessary for thorough microbial screening and the potential to move the safety criteria further toward the islets.<sup>44</sup>

This design supports the use of animals that have spent their adult life in the same high biosecurity facility, employing protocol-driven husbandry practices to provide donor animals of high health. The use of aseptic techniques and standard operating procedures from the procurement of the pancreas through to the isolation and encapsulation of the islets ensures freedom from adventitious virus contamination. The microbiological safety program presented here screens numerous tissues from all pancreas donor and sentinel animals for more than 25 pathogens. When combined with the use of pre-screened media and porcine serum, this approach controls major routes of introduction for adventitious agents that could enter the macrobead production process. This is the emphasis of Checkpoints 1 and 2. Monitoring the source herd provides information on potential breaks in biosecurity that may warrant additional testing or the temporary halting of macrobead production (Checkpoint 1). This is also true of individual donor animal screening and in this case may necessitate the discard of any macrobeads produced from suspect tissue (Checkpoint 2). Any positive viral findings in the macrobeads, with the exception of PERV, would automatically result in the destruction of those macrobeads (Checkpoint 3). Finally, patient monitoring under Checkpoint 4 provides not only valuable information for potential



treatment options in the case of suspected pathogen transmission but also manufacturing decisions as well as feedback to Checkpoints 1-3 that may result in the modification of one or more checkpoints.

As with any medical procedure, there will always be risk with the transplantation of porcine islets. The challenge we face is to understand those risks such that they can be minimized to an acceptable level in light of the expected clinical benefits. The potential risk of viral transfer from the transplanted cells to the recipient has been the major safety concern. To address this issue, a testing rationale was developed to screen sentinel and donor animals for viruses that are present in the herd, are of special interest such as known zoonotic agents, or are known to infect swine in the USA. An initial characterization of the PERV infectivity status of the source herd was not performed given the unlikely ability, at least at that time, to eliminate these endogenous retroviruses and the growing conclusion that the risk of PERV transmission to human xenotransplantation trial participants is low.<sup>60-62</sup> Viruses excluded from screening, to date, are those that are not known to be present in the United States (eg, Japanese encephalitis virus), unlikely to be present in the source facility (eg, vesicular exanthema of swine virus), or unlikely to be found in the pancreas or to survive during extended macrobead culture (Porcine torovirus). Many of these same screening exclusion criteria can apply to other infectious agents (eg, *Toxoplasma gondii* is unlikely to be present in the biosecure source herd). This is similar to the rationale used for pathogen screening as reported by Wynyard et al for the Auckland Island strain of swine<sup>63</sup> in which agents present or with an uncertain status in New Zealand, or the relevance in a biosecure facility or infectious potential post-transplantation, or the ability of the agent to survive in the environment of the pancreas were used to develop a list of screened pathogens. This herd, which originated from animals isolated on a remote island south of New Zealand, may be considered DPF because they are routinely screened and documented to be free of specific infectious bacteria, fungi, protozoa, and viruses. Although the rationale behind the development of this pathogen list was similar to our approach, the applications of the screening programs differ. Ultimately, the New Zealand group tested the source animal colony for 15 viruses, 10 bacterial species, and one protozoa. A reduced number of pathogens were screened for in the donor piglets and islet preparations. The Göttingen minipig herd in Ellegaard, Denmark, also qualifies as a DPF source animal facility as these animals have been shown to be free of at least 88 infectious agents including 20 viruses through PCR methodology. The one exception was the finding of swine Hepatitis E virus in 3 sow-piglet pairs and in 3 animals under one year of age.<sup>52</sup> These data suggest that in this case it may be possible to select donor animals based on the viral profile of individual animals.

Our approach favors routine screening of the donor herd with a greater emphasis on the screening of sentinel and donor animals. An even greater emphasis is placed on the qualification of the islet macrobeads (Checkpoint 3) in which an increased number of viruses are screened for using a variety of assays including specific PCR methods. Additionally, co-culture and electron microscopy assays are employed to look for unknown viruses, which would then prompt efforts to identify the virus as either a known or unknown agent (eg, PRRSV inducing

in MA104 cells as one component of the islet macrobead screen). Also, as part of their release criteria, the islet macrobeads are confirmed to be sterile and free from mycoplasma and any significant level of endotoxin. Although the current paper reports porcine islet macrobead screening results for the previous 2 years, we have found no evidence for the presence of known or unknown transmissible viruses in the islet macrobeads during screening of porcine pancreatic islets isolated from 30 donor animals over the previous 5 years. Moreover, no evidence of xenogeneic infection has been found in numerous tissues from animals implanted with the islet macrobeads.

New or emergent viruses are routinely investigated to optimize screening at the various checkpoints. For example, the presence of the PHoV (also known as porcine parvovirus) nucleotide sequence was investigated in source animals and in islet macrobeads. To date, this virus has not been cultured or isolated and the only evidence of its existence are the findings of the genomic sequence.<sup>64,65</sup> We have found evidence of the PHoV sequence in various porcine tissues including pancreas, mesenteric lymph nodes, donor serum, and frozen vendor supplied porcine serum used during islet isolation and macrobead culture. This sequence was not found in purified islets isolated in the absence of porcine serum. Because we also detected the sequence in domestic and international porcine serum sources, PHoV screening of islet macrobeads cultured in these sera was postponed until a suitable PHoV-free source could be obtained.

With regard to PERV, the identification of this virus and its ability to cross the species barrier has been the principal concern with porcine xenotransplantation. It is worth mentioning that the NHP is not an ideal model to assess the risk of PERV transmission. Cynomolgus monkeys lack the PERV-A receptor 1 (PAR-1) that is the major receptor for PERV-A and PERV-A/C entry.<sup>66</sup> The actual risk of PERV infection in the clinic remains unclear, but so far there has been no transmission of PERV or other porcine microorganisms in humans that have been exposed to pig islet cells.<sup>63,67-69</sup> We consider these findings, as well as the negative viral findings from diabetic dogs exposed to porcine islet macrobeads for 2.4 years, to be evidence that viral transmission following macrobead implantation is very low risk.<sup>15</sup> Studies with D17 canine cells have shown these cells to have the PERV-A receptor,<sup>70</sup> but apparently not to the same degree as human 293 cells.<sup>71</sup> Thus, there may be some question as to the relevance of the dog model for PERV transmission studies, although canine cells and subjects are susceptible to other porcine viruses including TGEV,<sup>72,73</sup> PRV,<sup>74</sup> and SIV<sup>75-77</sup> among others.

Other, non-microbiological risks to the patient also exist and must be considered as part of the comprehensive safety evaluation. These include procedural risks that accompany pre- and post-implantation screening, as well as the general anesthesia and laparoscopic surgery required to implant the macrobeads. The procedural risks are well known and, although real, are modest. We have been implanting cancer macrobeads (encapsulated mouse renal adenocarcinoma cells) to treat patients with various malignancies for 10 years without adverse events attributable to the macrobeads either as an intraperitoneal irritant or as a microbiological hazard.<sup>78</sup> See also ClinicalTrials.gov Identifiers NCT00283075, NCT01053013, and NCT02046174.

A final important consideration is the immune status of the recipients. Recent progress in clinical islet allotransplantation has confirmed the potential of islet grafting to reduce exogenous insulin administration and the occurrence of hypoglycemic unawareness.<sup>6,7</sup> While these results are encouraging, numerous adverse events including intrahepatic bleeding, neutropenia, mouth ulcers, anemia, diarrhea, edema, hypercholesterolemia, and pharyngitis have been reported.<sup>79</sup> Most of the reported adverse events are primarily associated with immunosuppressive therapy. Encapsulating porcine islets using the agarose-agarose method eliminates the need for immunosuppressive therapy, and a fully competent immune system is expected to significantly reduce the risk of xenozoonotic infection.<sup>80</sup> Likewise, it is generally agreed that non-genetically modified Source Animals reduce the risk of zoonotic infection because unmodified viral particles would be more likely to activate human complement-mediated destruction.<sup>81–83</sup> In our non-immunosuppressed streptozotocin-induced diabetic NHP surveillance, there has been no evidence of viral infection with PCV (Type 2), PLHV-1, 2 and 3, PRRSV, PCMV, or PERV in samples collected longitudinally post-transplant. Together, these data demonstrate a reduced risk in the absence of immunosuppression and suggests that the viral safety profile is acceptable for agarose encapsulated porcine islets.

During the last 20 years, an understanding of the risks associated with porcine xenotransplantation, and methodologies to manage those risks, including theoretical risks and the potential presence of unknown pathogens, have significantly progressed. The recent IXA consensus update notes that “theoretical risk” still exists in relation to the transmission of infectious agents when employing appropriate safeguards, but that such events will likely be rare should they occur.<sup>44</sup> Although the actual risks of islet xenotransplantation cannot be not known in the absence of clinical trials, as stated by Fishman, “In clinical xenotransplantation, a level of safety has been developed beyond that available for human organs...”.<sup>84</sup> Islet xenotransplantation is now at a point where clinical trials in the United States and Europe can once again be considered in light of the expected patient benefits. One particularly important benefit, based on our preclinical animal studies and islet transplantation in general, is the likely reduction in glycemic excursions that can be life threatening and that are experienced too often by patients with hypoglycemic unawareness. The microbiological safety program presented here screens a comprehensive set of tissues from all pancreas donor and sentinel animals for numerous infectious agents and, when combined with the macrobead screening assays, provides an unparalleled level of safety. These results support the use of a multicheckpoint biological safety program in the screening of encapsulated porcine islets intended for clinical trials for the treatment of hypoglycemic unawareness or unstable disease in patients with type 1 diabetes.

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## AUTHOR CONTRIBUTIONS

LSG, JC, AL, MJM, DG, HVV, MMM, DH, SR, RDH, and BHS performed the concept/design. LSG, JC, AL, RWH, DG, MG, MAL, DGM, and BHS performed the critical revision of article. RWH, MAL, CM, JB, EWM, DGM, and MMM performed the data collection/organization. All authors performed data analysis/interpretation, drafting the article, and approval of the article.

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