ORIGINAL ARTICLE

WILEY Xenotransplantation

Pathogen elimination and prevention within a regulated, Designated Pathogen Free, closed pig herd for long-term breeding and production of xenotransplantation materials

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Abstract

Background: We established a Source Animal (barrier) Facility (SAF) for generating designated pathogen-free (DPF) pigs to serve as donors of viable organs, tissues, or cells for xenotransplantation into clinical patients. This facility was populated with caesarian derived, colostrum deprived (CDCD) piglets, from sows of conventional-specific (or specified) pathogen-free (SPF) health status in six cohorts over a 10-month period. In all cases, CDCD piglets fulfilled DPF status including negativity for porcine circovirus (PCV), a particularly environmentally robust and difficult to inactivate virus which at the time of SAF population was epidemic in the US commercial swine production industry. Two outbreaks of PCV infection were subsequently detected during sentinel testing. The first occurred several weeks after PCV-negative animals were moved under quarantine from the nursery into an animal holding room. The apparent origin of PCV was newly installed stainless steel penning, which was not sufficiently degreased thereby protecting viral particles from disinfection. The second outbreak was apparently transmitted via employee activities in the Caesarian-section suite adjacent to the barrier facility. In both cases, PCV was contained in the animal holding room where it was diagnosed making a complete facility depopulation-repopulation unnecessary.

Method: Infectious PCV was eliminated during both outbreaks by the following: euthanizing infected animals, disposing of all removable items from the affected animal holding room, extensive cleaning with detergents and degreasing agents, sterilization of equipment and rooms with chlorine dioxide, vaporized hydrogen peroxide, and potassium peroxymonosulfate, and for the second outbreak also glutaraldehyde/ quaternary ammonium. Impact on other barrier animals throughout the process was monitored by frequent PCV diagnostic testing.

Result: After close monitoring for 6 months indicating PCV absence from all rooms and animals, herd animals were removed from quarantine status.

Conclusion: Ten years after PCV clearance following the second outbreak, due to strict adherence to biosecurity protocols and based on ongoing sentinel diagnostic monitoring (currently monthly), the herd remains DPF including PCV negative.

1 | INTRODUCTION

Xenotransplantation is defined as "Any procedure that involves the direct transplantation, implantation, or infusion into a human recipient of live cells, tissues, or organs from a non-human animal source; or indirect exposure, where human body fluids, cells, tissues, or organs that have had ex vivo contact with live non-human animal cells, tissues, or organs are administered".[1] Interest in xenotransplantation into humans arose when it became increasingly clear that the demand for transplants far exceeds supply. There is general consensus that the porcine species is the species of choice, based on a number of factors including that in many organ systems, the physiology of pigs and humans is remarkably similar. Pigs can be bred and maintained in large numbers within enclosed, biosecure facilities, with large litter sizes being in order of ten piglets or more, time to sexual maturity being short (about 6-8 months), and gestation time being less than 4 months. Genetic engineering techniques have been developed and are being used to modify pigs via transgenesis, gene modification, and gene knock-out (KO) technologies[2,3] to reduce immunogenicity and potentially improve safety. In our society, it appears to be ethically acceptable to use pigs as a source of cells, tissues, and organs for medical therapies.[4-6] As a result of these factors, a porcine-derived replacement pancreatic islet cell xenotransplantation product has now been used in patients with diabetes under national regulatory oversight[7-9] demonstrating both safety and efficacy.[10]

There are stringent guidelines issued by regulatory agencies including the US Food and Drug Administration (FDA) regarding the generation and production of pigs for Xenotransplantation.[11] Originally, the term specific (or specified) pathogen free (SPF) as it relates to swine production was not specifically created to help researchers but for pork producers to economically raise healthier animals under the premise that if the disease load can be lightened, better health and growth performance can be achieved.[12] However, higher level and more extensive pathogen exclusion standards have been proposed for designated pathogen free (DPF) herds,[11] with the notable exception of Porcine Endogenous Retrovirus (PERV), which due to its ubiquitous presence in the swine genome is risk managed via recipient monitoring,[11,13,14] subtype, and transmission potential characterization,[14] and recently potentially via genomewide inactivation.[3] Animals fulfilling DPF status need to be free of infectious pathogens, not only those that affect the health of swine populations, but also those known and unknown agents that have the potential to infect humans and cause infectious disease, that is, having xenozoonotic potential.[11] Examples of the latter category of potential pathogenic agents are porcine lymphotropic γ-herpes virus, hepatitis E, and porcine cytomegalovirus.[13,15] While no globally standardized DPF excluded agent list exists, there are a number of reports in the literature presenting lists of pathogens that should be eliminated from a DPF herd.[16-18] To enable this pathogen exclusion, DPF-free swineherds should be genetically closed and reared within biosecure barrier facilities, so-called Source Animal Facilities (SAFs),[11,18] located distant from any other swine production or transport routes, and isolated from potential pathogen sources via filtered air, filtered and disinfected water, sterilized feed, shower-in and shower-out access for staff, and hygienic measures including thorough screening of employees for potentially transmissible pathogens prior to hiring (Table 1). To reduce the risk of prion-based diseases, feed and feed components should be traceable and certified free of banned animal proteins or other cattle-derived materials for multiple generations prior to donation.[11] The operations in such a SAF should be according to current Good Manufacturing Practices (cGMP) conditions.[11] It is also proposed that SAF animal husbandry conditions should follow those for research animals per *the Guide for the Care and Use of Laboratory Animals*[19] with research facility registration and accreditation by the United States Department of Agriculture (USDA, in the USA), the Association for Assessment and Accreditation of

TABLE 1 List of diseases and infections screened for in animal contact personnel

Methicillin-resistant Staphylococcus aureus (MRSA)
Tuberculosis
Hepatitis A
Hepatitis B
Hepatitis C
Hepatitis E
Toxoplasma
Strongyloidiasis
Giardiasis
Round worm
Fecal Ova
Leptospirosis

Laboratory Animal Care International (AAALAC) respectively, and conduct of experiments under oversight of an Institutional Animal Care and Use Committee (IACUC).

One of many agents on established DPF exclusion lists is porcine circovirus (PCV). This is a small, nonenveloped, single-stranded DNA virus, first identified in 1974 as a contaminant of the porcine PK-15 kidney cell line, and it is the smallest known freely replicating virus in vertebrates.[20] There are two major strains, PCV1 and PCV2[21] along with a newly recognized third strain PCV3 whose significance is still being discovered.[22] PCV1 is not associated with disease, while PCV2 is associated with a spectrum of disease symptoms called porcine circovirus-associated disease (PCVAD)[20,21] first recognized as post- weaning multisystemic wasting syndrome. Clinical signs include progressive weight loss, lethargy, dark-colored diarrhea, lymphadenopathy, paleness, and jaundice. The hallmark lesion of PCV2 infection is lymphopenia in the blood circulation and lymphodepletion in lymphoid organs in which lymphoid cells are replaced by histiocytes with intracytoplasmic inclusion bodies. Cofactors in disease progression are multifold as PCV2 is immunosuppressive[23] and include the composition of the virus itself; coinfection by viruses such as porcine parvovirus and porcine reproductive and respiratory syndrome virus, and bacteria such as *Mycoplasma hyopneumoniae*[24,25] and host factors such as the breed of pigs, for example, purebred Landrace pigs being more susceptible than Duroc or Large White pigs.[26] Both types of PCV have a widespread global presence in swineherds: Antibodies to PCV1 and PCV2 were detected in archived pig sera taken as early as the 1970s, although the first clinical disease outbreaks of PCV2 were first reported in the second half of the 1990s.[21] The major route of transmission is via oral-nasal contact with infected feces, urine, or with infected pigs, but the possibility of vertical transmission through the placenta has been reported in field studies[27-29] and in experimental infection studies.[30,31] PCVAD is a globally established disease with a huge impact on pig production prior to widespread vaccine availability. PCV2-specific vaccines became commercially available during the mid-2000s and proved efficacious in field trials.[21] In our studies, we used a capsid-based subunit vaccine marketed in North America under the name Circumvent® (Intervet Inc/Schering-Plough Animal Health, Boxmeer, the Netherlands) that first became available in limited quantities during 2007. This vaccine showed efficacy in experimental studies[32] and field investigations, that is, a reduction in mortality by 50% and increased weight gain upon vaccination of piglets at weaning and 3 weeks later.[33] In one experimental study, effectiveness of immunization of the dam was investigated: Despite formation of antibodies after immunization at 4 weeks of gestation, virus inoculation at 8 weeks did not prevent transmission to the fetus.[34]

Importantly, PCV is extremely robust and resistant to disinfection procedures. It is stable at pH 3 and resistant to heat inactivation even up to 120°C for 30 minutes.[24] It is also resistant to inactivation by chloroform.[35] PCV2 appears more resistant to disinfection than PCV1 where pasteurization for 10 hours at 60°C and dry-heat treatment for 72 hours at 80°C had some effect, but no effect was observed for 30-minute dry-heat treatment at 120°C; however, wet-heat treatment up to 80°C proved efficacious in virus inactivation.[36] Experimentally, effectiveness in virus titer reduction was observed for disinfectants such as Virkon®-S, sodium hydroxide, 3%-6% sodium hypochlorite (bleach), Roccal D Plus®, 1-Stroke Environ®, Fulsan®, and Tek-Trol®; however, no effectiveness was observed for Nolvasan®, Neogen DC&R®, Weladol®, or ethanol.[37] Bleach is effective, but it is not known whether its use is effective in the field.[38] Iowa State University has published a disinfection protocol to be used in disinfection of pens, which starts with a degreaser detergent followed by decontamination with Virkon®-S (Antec International, Sudbury, Suffolk, UK) at 1:30 dilution and then fogging with Clidox®-S (US Pharmacal Com LLC, Erie, CO) at 1:5:1 dilution.[21]

Porcine circovirus is on the list of DPF pathogens of exclusion mainly because of its potential impact on the health of pig herds. Its zoonotic potential for man has not been clearly established, as circoviruses have been found in other species but not in humans.[39] Exposure in humans has been claimed by serological testing for antibodies: About 20% of healthy adults and 30% of hospital patients with fever of unknown etiology were found to be antibody positive,[40] but this has not been confirmed in other studies.[41] Also, antibodies to PCV1 or PCV2 proved not detectable in a population of professionals with frequent, close contact to pigs, that is, veterinarians in swine practice.[42] There are indications that the circovirus can infect some selected human cell lines during in vitro coculture, but this infection is non-productive[43] for primary human blood mononuclear cells, and there is conflicting data on susceptibility to in vitro infection.[44,45] From these data, it appears that the risk of zoonosis of PCV is rather small. In non-human primate recipients of a porcine pancreatic islet product, there was no evidence observed for transmission of PCV[46] and also patients who received clinical porcine islet transplants did not show evidence for PCV.[14,47] However, it is not clear in the latter study whether the source pigs were PCV positive. The issue of potential cross species transmission received attention after DNA from PCV1 was reported in rotavirus vaccines prepared independently by two pharmaceutical companies: in one of these, DNA from PCV2 was also detected. After consideration of this finding, regulatory authorities in Europe[48] and USA[49] concluded that due to minimal risk of zoonotic potential, this DNA presence does not pose a safety concern and that there is no need to restrict the use of these vaccines.

Confirmation of PCVAD/PCV2 depends on a combination of clinical signs, characteristic gross and microscopic lesions, and detection of the virus in the tissues. Quantitative polymerase chain reaction assay (qPCR) is useful in the identification and quantification of PCV2 and PCV3 in tissues or serum. The virus threshold which suggests disease causality varies significantly between laboratories, and qPCR alone is not sufficient for individual diagnosis. Immunohistochemistry (IHC) and in situ hybridization are utilized to detect PCV2 in tissues and used in conjunction with qPCR.[50] Environmental samples taken using swabs have been used to detect environmental PCV viral DNA using PCR.

TABLE 2 Founder animal cohorts used to populate the SAF

Cohort ID	Nr of sows	Total nr of piglets derived	Nr of piglets deceased within 3 weeks after birth	Cause of death
1	3	26	5	Navel rupture, umbilical clamps lost
2	3	27	4	Runts
3	6	41	16	Bacterial infection
4	3	23	13	Bacterial infection
5	3	30	12	Bacterial infection
6	2	18	5	Bacterial infection

2 | POPULATION OF A SOURCE ANIMAL FACILITY

The population phase of SAF operation poses the highest risk of introduction of a pathogen. In the rare instance that an SAF is populated with a DPF source of animals such as was the case for the population of the Living Cell Technologies SAF in New Zealand using South Auckland island pigs that were DPF by virtue of geographic isolation on a remote island, this risk is reduced, but not eliminated. Another potential example of this situation would be the population of a new SAF using founder animals from an existing SAF. In such cases, pathogen exposure during transport for population poses the highest, but logistically manageable, risk. However, the animals themselves, by virtue of their intrinsic high health status, do not pose a significant risk. Furthermore, in such instances, population can be effected using founder animals of any life stage including adults with fully developed, albeit naïve, and immune systems.

In our situation, we were tasked with populating a new, unoccupied SAF with DPF founder animals derived from existing SPF herds based on the criteria that certain domestic swine breeds have been found to possess a higher propensity for high islet of Langerhans yields from adult donors.[51] Selection of founder animals was not based on PERV C negativity, but a proportion of the founder animals were subsequently found to be PERV C negative.

2.1 | Origin of animals for SAF population

Our SAF was populated with CDCD piglets derived from vaccinated SPF sows originating from a multiplier unit. The initial population of this multiplier unit was performed in June 2005-July 2006 using founder animals selected from conventional, commercial breeding herds.

The multiplier unit was located at about 1.5 miles distance from a commercial swineherd, and despite typical commercial biosecurity measures like shower-in shower-out performance, a number of pathogens emerged in the herd. During 2006, this included *Staphylococcus aureus*, *Staphylococcus hyicus*, *Actinobacillus pleuropneumonia*, *Enterococcus*, *Clostridium perfingens* and *Clostridium difficile*, *Cryptosporidia*, various *Salmonella* species, *Leptospira Icterohaeemorrhagiae*, *Hemagglutinating Encephalomyelitis* Virus, *Adenovirus*, and PCV. The first PCV-positive result was reported in a sample taken in August 2006, about 6 months before the first cohort of piglets was planned to be derived at the SAF.

All sows at the multiplier unit that were used for SAF population were vaccinated for porcine parvovirus (PPV) and Leptospira, using Farrowsure plus *Leptospira bratislava* (Pfizer Animal Health, New York, NY), at approximately 6 months of age with a booster 3-4 weeks later and with an attempt to time this vaccine 2-3 weeks prior to initiation of pregnancy by artificial insemination.

A PCV vaccine was not available in the United States prior to population cohort #4 (Table 2). After we received a PCV2 vaccine (Circumvent®, Intervet), the sows used for population cohort #4 and beyond were vaccinated, with a booster administered 2-3 weeks later. To decrease the possibility of PPV and Leptospirosis entering the barrier facility during population, pregnant sows were assessed for antibody titers to PPV and Leptospirosis close to initiation of pregnancy. PCV viremia was also checked at this timepoint by PCR testing. PCV viremia monitoring via PCR was then performed repeatedly during the gestation period. If a sow exhibited antibody titers to PPV or Leptospirosis or was viremic for PCV at any point during gestation, the sow was excluded. Once initiated, the PCV vaccination program greatly reduced the level of PCV viremia in the multiplier unit, increasing the available number of suitable sows for SAF population. All C-section derivations conducted at the SAF successfully removed all DPF pathogens and the prevented pathogen entry, that is, infectious agents present in the sow were prevented from being transmitted to the CDCD founder piglets.

2.2 | The SAF

Our organization has constructed and operates a DPF SAF facility.[52] SAF design was initiated in 2005, followed by construction of the $5.7 million USD facility in 2006, and subsequent population in 2007. Although the use of killed vaccines in SAFs is permitted by the FDA, animals in the facility are not vaccinated due to the lack of pathogen exposure and the potential diagnostic interference prophylactic vaccination poses, and antibiotic use is minimal. The facility is registered and regularly inspected by USDA and has received full accreditation by AAALAC.

The floor plan of the SAF is outlined in Figure 1 with the typical animal census ranging from 50 to 100 pigs depending on their age and size. It encompasses 21,192 ft^2 in total of which 13 977 ft^2 is

FIGURE 1 Floor plan of the SAF facility indicating cohort #1 founder population flow. The barrier is bordered with blue lines, and flow of the sow and initial cohort of piglets though the building is indicated, [continuous red line] from C-section in the Operating Room (OR) to quarantine nursery and [interrupted red line] CDCD piglets from quarantine nursery to animal holding rooms

FIGURE 2 Floor plan of the SAF facility indicating cohort #2-6 founder population flow. The barrier is bordered with blue lines, and the location of the external C-section suite is shown. The flow of the piglets though the building is indicated, [continuous red line] from C-section in the external C-section suite to quarantine nursery and [interrupted red line] CDCD piglets from quarantine nursery to holding rooms

animal care and use (barrier) space. Animal space includes eight approximately 772 ft² rooms (one quarantine nursery/grower room, five finisher rooms, one farrowing room, and one boar room), each of which has a Danish entry and is ventilated by completely separate, single-pass filtered air flow under either positive or negative differential pressure relative to adjoining spaces depending on need. Water supply is via an on-site deep well supplying filtered, UV-sterilized water. Entry to the barrier is via a vaporized hydrogen peroxide (VHP) fume room for heat labile supplies and feed, or via a pass-through autoclave for durable supplies. VHP was also used to thoroughly disinfect the SAF barrier prior to the delivery of the first founder piglets. A controlled access locker room and shower-in/shower-out facilities are present for personnel entry and exit. Support rooms outside the barrier include surgical and necropsy suites, animal holding, laboratory, and office space. The short-term animal holding room outside of the barrier and surgical

and necropsy suites were only used for the delivery of the first cohort of piglets, in which the C-sections were conducted in the surgical suite, and animals delivered via an air lock into the barrier (Figure 1). For subsequent cohort deliveries, an external Caesarian-section suite located directly adjacent to but separate from the SAF was used. When this suite was used, CDCD piglets were aseptically transferred to the SAF barrier in sealed, sterile containers in which they were transported directly into the quarantine nursery as indicated in Figure 2 to reduce contamination risk.

2.3 | SAF population

In total, 6 cohorts of pregnant sows were transported from the multiplier unit for SAF population. These sows were euthanized after the caesarian sections and never entered the barrier facility. The

TABLE 3 Designated Pathogen Free (DPF) listing of excluded pathogens

Bacteria:

- *Actinobacillus pleuropneumonia*
- *Actinobacillus suis*
- *Bacillus anthracis*
- *Bordetella bronchiseptica*
- *Brucella sp.*
- *Campylobacter sp.*
- *Chlamydia sp.*
- *Erysipelothrix sp.*
- *Haemophilus parasuis*
- *Lawsonia intracellularis*
- *Leptospira sp.*
- *Mycoplasma hyopneumonia*
- *Mycoplasma hyorhinis,*
- *Mycoplasma hyosynoviae*
- *Mycobacterium tuberculosis*
- *Mycobacterium bovis,*
- *Mycobacterium avium*
- *Pasteurella multocida*
- *Pasteurella. haemolytica*
- *Salmonella sp.*
- *Brachyspira sp.*
- *Staphylococcus hyicus*
- *Streptococcus suis*
- *Yersinia sp.*

Fungi:

- Systemic mycoses including:
  - *Blastomyces sp.,*
  - *Cryptococcus sp.,*
  - *Histoplasma sp.*

Parasites:

- Pathogeneic Protozoa including:
  - *Cryptosporidium parvum,*
  - *Giardia sp. and*
  - *Toxoplasma sp.*
- Helminths
- *Trichinella spiralis*
- Blood parasites

Arthropods:

- All pathogenic arthropods (eg lice and mites)

(Continues)

TABLE 3 (Continued)

Viruses:

- Adenovirus (Porcine)
- Bovine Viral Diarrhea Virus
- Porcine Circovirus types 1 and 2
- Encephalitis, Eastern and Western Equine
- Encephalomyocarditis Virus
- Enterovirus
- Hemagglutinating Encephalomyelitis Virus
- Hepatitis E
- Infectious Bovine Rhinotracheitis Virus
- Swine Influenza Virus
- Porcine cytomegalovirus
- Porcine Parvovirus
- Porcine Reproductive and Respiratory Syndrome Virus
- Parainfluenza 3 Virus
- Pseudorabies Virus
- Porcine Respiratory Coronavirus
- Rotavirus
- Transmissible Gastroenteritis Virus
- Vesicular Stomatitis Virus (NJ & Indiana)
- West Nile Fever Virus
- Porcine Lymphotropic Herpes virus 1 and 2

resulting CDCD piglets were delivered into the facility (Table 2) between February 2007 and November 2007. The CDCD piglets were housed for the first 3 weeks in the quarantine nursery in isolated nursery carts outfitted with tenderfoot flooring. After 3 weeks, the animals were moved into animal holding rooms outfitted with stainless steel penning affixed to epoxy sealed concrete flooring and walls. The mortality of newborn piglets during the first 3 weeks varied for the various cohorts between 15% and increasing to a maximum of 57%. This mortality increase was despite intensive management and increased experience in caring for CDCD neonates during the population phase and was ascribed to infections by non-pathogenic microflora in the facility that accumulated during the initial facility operations and the absence of natural immunity of CDCD piglets (being colostrum deprived). To replace dam's

colostrum and milk, piglets were fed Esbilac® liquid (a Rendered and Recycled Mammalian Material (RRMM)-free synthetic milk replacer manufactured by PetAg, Inc., Hampshire, IL) from birth until weaning. In addition, a probiotic containing a pure mixture of *Lactobacillus acidophilus* (7 500 000 CFU/dose) and *bifidobacterium* (22 500 000 CFU/dose; Danisco, Madison, WI) was orally administered every other day (7 doses total) during the first 2 weeks. All animals then received Harlan-Teklad Pre-Starter ration from day ~17 until the post-weaning period followed by Harlan-Teklad Starter ration and Harlan-Teklad Grower ration until 14-16 weeks of age and then were maintained on Harlan-Teklad Maintenance ration. The rations fulfilled the regulatory requirements for the absence of RRMM proteins in place at the time of the 2003 version of the FDA regulatory Guidance, pesticides, and herbicides.

All founder animals were assessed for their DPF status (Table 3) before being released from quarantine, and in all cases, the DPF status including the absence of PCV was confirmed.

These population procedures were conducted successfully, and all CDCD founder piglets fulfilled DPF criteria. However, during the population phase, we were confronted with two separate outbreaks of PCV within the SAF. We here expand on specific activities undertaken to identify the cause and contain these outbreaks, so that the introduced virus was completely eliminated from the SAF and its herd.

During SAF population, animals were frequently assessed as part of a surveillance program for pathogen entry even in instance of subclinical symptomology in the new facility. During the first months of the population, frequent testing was made on serum samples, swabs, and tissue samples taken from sentinel animals used for necropsies (full sentinels) and piglets that died or were humanely euthanized due to health issues. This rigorous surveillance program led to the rapid discovery of PCV outbreak #1.

2.3.1 | PCV outbreak #1

The first SAF outbreak involved cohort #1. All piglets were PCV negative upon release from quarantine nursery at 3 weeks of age when they were moved, still under quarantine, into an empty animal holding room. At 4 weeks of age, PCV was detected in sentinel serum and tissue via PCR. Subsequent intense environmental surveillance of the entire facility for PCV contamination was performed using swabs and PCR. This resulted in the detection of PCV in grease residue that remained on penning in another barrier animal holding room that had been disinfected but had not yet been populated. An adjacent room had been similarly disinfected, but the penning had then been manually scrubbed with water containing 10% bleach to "polish" the stainless steel resulting in no detectable PCV contamination. We concluded that the cause of infection was an ineffective cleaning/degreasing of stainless steel penning that apparently then harbored and protected the PCV particles from disinfection. Furthermore, it was found that the fabrication of the stainless steel penning occurred in close proximity to commercial swine production further bolstering our conclusion.

Cohort #1 was subsequently euthanized. All items that were not permanently affixed to the affected room were immediately disposed of, and the room was extensively cleaned with detergent and water to remove all feces and waste material. All penning in all animal rooms were then wiped with chlorine dioxide (Clidox®), degreased with a commercial degreaser, scrubbed with a detergent/10% bleach water solution, and then wiped with Clidox® again. Following this treatment, all animal rooms within the barrier were cleaned with water and then sequentially with the following: [1] potassium peroxymonosulfate (Virkon®-S or Trifectant®) foamed and allowed to dry on all surfaces prior to rinsing with water; [2] vaporized hydrogen peroxide (VHP) fumigation; and [3] fogging with Clidox®. Again, intense environmental surveillance was performed using swabs, all of which failed to return PCV-positive PCR results. As a precautionary measure, the outbreak affected room was left empty for 6 months prior to a next population. Live sentinel animals were then placed into quarantine in the affected room, and frequent monitoring for PCV was conducted on these sentinels for approximately 2 months prior to resuming the population of the room.

Coincident with the cleaning and disinfection, while awaiting the results of cleaning program effectiveness, and before the second cohort's CDCD derivation was initiated; a change in location for subsequent C-section derivations was installed. This was based on risk assessment which found it prudent to maintain potentially infectious sows outside of the SAF envelope for the subsequent C-section derivations. This modular external C-section suite was located directly adjacent to, but separate from the facility (Figure 2).

2.3.2 | PCV outbreak #2

After the cleaning of the facility described above, and using the external C-section suite, the delivery of piglets in cohorts #2, #3, #4,

and #5 was without incident. All animals fulfilled DPF status and were released from quarantine into animal holding rooms at 3 weeks of age. Animals in cohorts #4 and #5 were cohoused in one of the animal holding rooms. Five weeks after entry of animals from cohort #5 in this room, pigs were found to be PCV positive by ELISA, IHC, and PCR. The affected room was immediately put into quarantine, and its differential pressure adjusted to be negative to adjacent spaces to contain the outbreak. Once again, an extensive environmental surveillance of the SAF barrier was performed using swabs and PCR analysis. This resulted in the isolation of PCV DNA from a sample obtained by swabbing a section of the barrier storeroom. No other virus DNA-positive samples were found in the animal facility. Facility records were reviewed, and all staff were interviewed to assess their activities during the relevant time period of virus introduction and identification. These investigations led to the conclusion that the entry of PCV virus was likely via an employee who performed activities in the external C-section suite followed by entry into the barrier and the affected rooms. Furthermore, the genotype of PCV isolated from within the SAF matched the genotype of the source herd from the multiplier unit which in turn matched the genotype of PCV isolated from the external C-section suite.

Affected cohoused animals from both cohorts #4 and #5 were euthanized after the PCV diagnosis was confirmed. Members of cohorts #2 and 3# proved to be PCV negative and were maintained in their respective holding rooms within the barrier. Again, all items that were not permanently affixed to the affected room were disposed of. Extensive cleaning with detergent and water was performed to remove all feces and waste material. The room and penning were then foamed with potassium peroxymonosulfate which was allowed to dry on surfaces. This procedure was performed twice. Following this, the room was sterilized by chlorine dioxide fog, disinfected with potassium peroxymonosulfate foam and also with glutaraldehyde/quaternary ammonium (Synergize®) foam. Disinfectants were applied, allowed to dry overnight, rinsed with water, allowed to dry, and then alternate disinfectants applied. This was performed multiple times until testing of the room using swabs, and PCV via PCR analysis was repeatedly confirmed negative. The affected room was left empty for 6 months prior to repopulation during which time it was also regularly tested for PCV via PCR. At the end of this period, live sentinel animals were placed in the room and tested for PCV for a period of approximately 2 months. After proving that there was no PCV emergent in these animals, the quarantine status of the room was discontinued, and the room returned to non-quarantine positive differential pressure.

Coincident with this cleaning period, cohort #6 was successfully delivered into the barrier without incident or emergent pathogenesis.

Considering both the risks of pathogen introduction with subsequent cohort derivations and the extent of genetic diversity present in the animals introduced in cohorts #2 and #3, it was decided that further entry of new animals was not warranted beyond cohort #6, and that subsequent herd expansion would be by an internal breeding program. As a result, following the derivation of piglets from cohort #6, the herd has been genetically closed.

Currently, more than 10 years after the 10-month population phase of operations concluded the herd remains closed, PCV negative, and meets all other DPF requirements.

3 | SAF OPERATIONS IN THE POST-POPULATION PHASE

While the risk is reduced, it is only with great diligence and attention to biosecurity protocols that we are able to successfully maintain a DPF SAF in the post-population phase, albeit at an annual operational cost of nearly $2 million USD.

Hiring of personnel who will enter the barrier is contingent upon successfully completing extensive health screening (Table 1) to reduce the risk of personnel transmission of pathogens to the DPF herd. Included in the criteria is testing for Hepatitis E (HEV) which is an important public health concern and with which an estimated one-third of the world's population has been infected, most asymptomatically.[53] HEV can be transmitted through blood, hence the concern from staff working with the pigs or islets.[53] The xenozoonotic potential of HEV is of importance in DPF swine production as HEV can induce disease and chronic infection in immunosuppressed individuals.[54]

Annual tuberculosis and Methicillin-resistant *Staphylococcus aureus* (MRSA) testing are required along with influenza vaccination, and serum is archived for all staff with animal contact to facilitate investigation of any potential future outbreaks. Inspectors of the barrier for regulatory and accreditation authorities also require successful completion of health screening (Table 1) to access the barrier, or inspections are conducted remotely via a 2-way on-site video camera system.

Strictly enforced staff sick policies require extended absence for personal illnesses to assure the health of the swine population. Strict visitor and staff animal contact policies require a minimum of 72 hours withdrawal in the case of any swine exposure outside of the SAF.

Breeding is performed using semen collected from boars in the barrier and artificial insemination maintaining the closed herd and also reducing the risk of introduction of pathogens via semen.

The sentinel disease surveillance program continues to monitor the health of the swineherd and incorporates both full and live sentinels on a rotating monthly basis, which is a lower frequency than during the population phase due to decreased risk. Archived diagnostic samples from sentinels and deceased animal necropsies are archived for retesting purposes, for future assessment of currently unknown pathogens, and for investigation if needed. The DPF excluded agents' list has been refined as new, emergent diseases have been discovered, and validated tests for the pathogens responsible have been developed.

With this intense focus on biosecurity, we have been successful keeping the SAF herd DPF for over 10 years and activities are ongoing.

4 | DISCUSSION

While the design, construction, and operation of a SAF are all challenging, the population phase of SAF operation poses the highest risk for the introduction of a pathogen which, depending on the pathogen, can be manageable or devastating to program success. In our experience, despite successful initial introduction of DPF piglets from SPF sows via CDCD during the population phase of a new SAF, we were confronted with two separate PCV outbreaks. These outbreaks were not expected as the CDCD piglets upon release at three weeks of age from quarantine nursery were in all cases free of all DPF listed agents of exclusion. Both PCV outbreaks first occurred after piglets were moved into animal holding rooms. In the first outbreak, it was found that the means of transmission was via the stainless steel penning in animal holding rooms; the second via employee facilitated contamination from a PCV high risk area, the external C-section suite located adjacent to the SAF, which was then transferred by the employee's entry into the barrier and direct contact with PCV-negative, naïve pigs. In both instances, the outbreak was identified quickly and the source of the PCV was ultimately able to be determined, indicating that the diagnostics used were sensitive enough to detect PCV early in the course of infection.

The first outbreak represents an unusual circumstance but illustrates the extreme robustness of the PCV virus. The stainless steel penning was manufactured at a facility close to a commercial pig herd and employees at the stainless steel manufacturer also had close contact with the nearby swine farm. Some of these individuals also installed the penning with its rust-preventative grease in the barrier prior to its closure after construction. Apparently, in some rooms, the penning was then insufficiently degreased before disinfection, and intact PCV virus was thereby protected by the grease on the stainless steel allowing survival during disinfection using aqueous media. The ensuing physical contact of the piglets and the greasy penning likely then liberated the PCV and initiated the outbreak. The second outbreak was not a tremendous surprise: It was known that each cohort of C-section piglet derivations involved extensive set up, surgical activities, and clean up—each of which was a high risk activity. Careful planning was in place to minimize the potential for personnel to be a vector for transmission, but proved ineffective.

During the period of both PCV outbreaks, we did not observe any symptom of PCVAD, presumably because the frequent diagnostic testing program enabled the detection of PCV at an early phase post infection and likely also to the absence of other infectious agents which would have contributed to the disease load on the piglets.[23]

Of the many pathogens in the DPF excluded agents' list,[11,14,16,17] PCV was in 2007 a pathogen of most concern given its robustness, difficulty to inactivate, uncontrolled epidemic status in the United States with no vaccine available until early 2007, and its potential for vertical transmission in utero.[27,28] The barrier facility was populated at a time when PCV in the swine industry of the United States was considered ubiquitous and at an epidemic status causing great morbidity and mortality.[20,21] Half a year before the first piglets were delivered, PCV was first reported positive in the multiplier unit where

the donor sows were housed. Therefore, essentially during the phase of populating the DPF barrier, the herd in the multiplier unit was in a first phase of viral contact, active viremia, and seroconversion. Since the start of operations at the multiplier unit in 2005, we had not observed any animal showing PCVAD. Initially,we took no special precautions at the time to specifically prevent PCV entry into the barrier, but were successful in the case of cohort #1. After the first PCV outbreak, PCV-specific preventive measures were taken including testing of sows for PCV viremia at breeding and during pregnancy. Once a vaccine became available, starting with cohort #4, we initiated vaccination of sows used for producing founder animals before and during pregnancy. It is not clear whether vaccination was necessary, but the outcome was successful as none of the piglets delivered to the barrier facility were PCV positive immediately following introduction. Exacerbating the uncertainty of whether vaccination was a necessity are the facts that PCV vaccines are typically developed for first administration at an early life stage,[21,33] and that vaccination of pregnant sows does not completely prevent infection of piglets via the transplacental route.[34] However, the exclusion of sows exhibiting PCV viremia during pregnancy is supported by the fact that transplacental infection of fetuses is quite likely in such animals.[29,30]

The SAF was designed with separate animal holding rooms, each with its own UV-sterilized water supply, separate single-pass ventilation (with positive or negative differential pressure relative to adjacent spaces), and Danish entry. This compartmentalized, isolation enabling design facilitated successful containment of the outbreak, depopulation of only PCV infected animals in affected animal holding rooms, disinfection, and repopulation without requiring the depopulation of adjacent, unaffected rooms within the barrier. Interestingly, we obtained infrequent PCR-positive environmental swab sample results from the empty, quarantined rooms despite repeated cleaning and disinfection. However, when sentinel animals were brought in the room, and PCV did not emerge in these animals, it indicated that the PCR-positive results might have detected remaining DNA fragments from non-infectious virus. We concluded that the design of the facility, with completely separate and isolatable animal holding rooms, worked in this approach to contain a potentially devastating infectious outbreak and prevent its spread.

From our experience with outbreaks during the population phase of our DPF SAF at a time when one of the viruses on the DPF excluded agents' list was endemic and ubiquitous in almost every commercial swineherd, we concluded a number of general recommendations:

1. Populate a DPF facility in just one group of derivations if at all possible, which eliminates repeated exposure of the facility to high virus exposure risk activities;

2. Assume that everything that enters the facility may be virus-positive;

3. Take appropriate measures to disinfect all materials and supplies prior to population;

4. Test the environment repeatedly prior to population;

5. Continue to perform frequent testing throughout the quarantine period to catch outbreaks early and prevent spread.

We demonstrated that despite the presence of a high PCV infection risk during population it is possible to populate a PCV-negative DPF swineherd during a PCV epidemic. With the advent of widespread vaccination, the risk for PCV infection has diminished, but many of the risks and risk-reduction strategies we have described are relevant to not only PCV, but to other endemic or emergent swine pathogens on DPF excluded pathogen lists.

There have been a number of emergent diseases which have been identified since the establishment of our DPF SAF. Not all of them have a zoonotic potential but these diseases would definitely adversely impact the health of the DPF herd. Porcine Endemic Diarrhea (PED) emerged in the United States and caused an epidemic beginning in 2013 with very high morbidity and mortality in piglets and leading to the loss of more than 10% of the US pig population.[55] PCV3 is on the increase in adult pigs with much to be learned about its significance,[50] and Seneca Valley Virus (Senecavirus A) is of concern due to the similarity of lesions it causes to Foot and Mouth Disease.[56] The risk from a strain of influenza which can pass from people to pigs is ever present. African Swine Fever (ASV) is of growing concern in Eastern Europe and parts of Africa.

The care and biosecurity under which the SAF was populated and continues to be managed indicate that, while challenging, maintaining a clinically suitable herd of DPF pigs long term is feasible.

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