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Xenotransplantation panel for the detection of infectious agents in pigs

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Abstract

Background: Recent advances in xenotransplantation have produced organs from pigs that are well tolerated in primate models because of genetic changes engineered to delete major antigens from donor animals. To ensure the safety of human transplant recipients, it will be essential to understand both the spectrum of infectious agents in donor pigs and their potential to be transmitted to immunocompromised transplant recipients. Equally important will be the development of new highly sensitive diagnostic methods for use in the detection of these agents in donor animals and for the monitoring of transplant recipients.

Methods: Herein, we report the development of a panel of 30 quantitative polymerase chain reaction (qPCR) assays for infectious agents with the potential to be transmitted to the human host. The reproducibility, sensitivity and specificity of each assay were evaluated and were found to exhibit analytic sensitivity that was similar to that of quantitative assays used to perform viral load testing of human viruses in clinical laboratories.

Results: This analytical approach was used to detect nucleic acids of infectious agents present in specimens from 9 sows and 22 piglets derived by caesarean section. The most commonly detected targets in adult animals were *Mycoplasma* species and two distinct herpesviruses, porcine lymphotrophic herpesvirus 2 and 3. A total of 14 piglets were derived from three sows infected with either or both herpesviruses, yet none tested positive for the viruses indicating that vertical transmission of these viruses is inefficient.

Conclusions: The data presented demonstrate that procedures in place are highly sensitive and can specifically detect nucleic acids from target organisms in the panel, thus ensuring the safety of organs for transplantation as well as the monitoring of patients potentially receiving them.

KEYWORDS
coronaviruses, herpesviruses, Infectious disease panel, virus safety of xenotransplantation

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1 | INTRODUCTION

Continued shortages of organs for transplantation have limited the number of transplants each year, and many patients die before organs become available. Transplantation of porcine organs has become an increasingly attractive approach as CRISPR/cas9 genome editing techniques have succeeded in reducing the rejection rate of transplanted organs.[1] Transmission of infectious disease during transplantation is always a concern, and allogenic transplantation is associated not only with the reactivation of opportunistic infections from the recipient, but also infectious agents present in the donor tissue that is transmitted to the immunosuppressed hosts.[2,3] In the case of xenotransplantation, transmission of zoonotic infections, such as hepatitis E virus (HEV), or porcine infectious agents that might gain access to the human host during transplantation is an additional concern.[4,5] At present, the full spectrum of infectious agents with the potential for transmission to the human host, or that might produce pathology in transplanted porcine organs, has not been completely defined, but will improve over time. Going forward, ensuring the safety of these organs is of paramount importance, and the use of highly sensitive techniques to detect potential pathogens will help to minimize adverse events in xenotransplantation.

The highly sensitive methods used for the detection of human viruses in transplant recipients have been applied to the detection of porcine viruses in pigs and has been recently reviewed.[6] Notable examples include the detection of porcine cytomegalovirus (PCMV)[7,8] and hepatitis E virus (HEV)[9,10] using quantitative polymerase chain reaction (qPCR). The sensitivity and performance of these analytical methods appear to be comparable to those used in the clinical setting to detect opportunistic infections in human transplant recipients, and indeed, these assays promise to characterize porcine samples at a much more rigorous level than that used qualify tissues from human donors.[3]

The studies described here are part of a larger effort to generate genetically engineered porcine organs for use in clinical trials to evaluate the potential of xenotransplantation.[11-13] Specifically, a set of 30 targets including viruses and *Mycoplasma* species was selected to include agents that have potential to impact the health of the graft tissue, or with the potential to be transmitted to humans during transplantation. Herein, we describe a diagnostic approach to detect a broad spectrum of infectious agents by adapting primer and probe sets reported in the literature to conform to a common amplification protocol on a common instrument. This new testing regimen was then utilized to detect each of the targets in 9 sows and 22 piglets derived by cesarean section.

As many of the agents in this set are expected to be present in distinct tissues, blood, nasal swabs, and stool swabs were evaluated to enhance their detection. These data confirmed the presence of viruses that are endemic in the pig population in the United States, and this approach will be useful in helping to establish the frequency of many infections in herds. Finally, the data show that piglets derived

by cesarean section were not infected with the same agents detected in the sows from which they were derived and are consistent with previous reports for herpesviruses.[14,15]

2 | MATERIALS AND METHODS

2.1 | Synthesis and characterization of control plasmids

To validate the infectious disease testing assays, a set of positive control plasmids was designed and synthesized to provide target sequences for each of the reactions. A description of sequences targeted by each of the assays is shown in Table S1 and includes the sequence of all the primers and probes, the accession numbers from cognate agents, and genomic coordinates. Each plasmid contains multiple targets as well as sequences for herpes simplex virus (HSV) to facilitate the absolute quantification of each plasmid by a validated assay in a clinical laboratory by established primers and methods.[16] Plasmids were synthesized, cloned, and sequenced by a commercial supplier to yield qualified stocks of target sequences (Integrated DNA Technologies, Coralville, IA). Target sequences were concatenated to produce a single DNA sequence from multiple organisms, synthesized, and cloned into pUCIDT to yield control clones carrying the following sets of target sequences: pMP638, HSV, porcine circovirus type 1 (PCV1), porcine circovirus type 2 (PCV2), and porcine parvovirus (PPV); pMP639, HSV, norovirus genogroup II (Noro2), West Nile virus (WNV), bovine diarrhea virus (BVDV), porcine hemagglutinating encephalomyelitis virus (PHEV), and transmissible gastroenteritis virus (TGEV); pMP640, HSV, encephalomyocarditis virus (EMCV), seneca valley A virus (SVA), porcine reproductive, and respiratory syndrome virus (PRRV); pMP645, HSV, HEV, reovirus 3 (REO3), porcine lymphotropic herpesvirus 1 (PLHV1), and porcine lymphotropic herpesvirus 3 (PLHV3); pMP646, HSV, porcine lymphotropic herpesvirus 2 (PLHV2), porcine astrovirus (ASTV), sapovirus (Sapo), and human influenza B virus (IFB); pMP647, HSV, PCMV, porcine adenovirus (PAdV), *mycoplasma haemofelis* group (MycoF), *mycoplasma haemominutum* group (MycoM), porcine endogenous retrovirus C (PERV-C), and chikungunya virus (CHIKV); and pMP652, HSV lymphocytic choriomeningitis virus (LCMV), rabies virus (RabV), swine influenza (IFA), reovirus 1 (Reo1), reovirus 2 (Reo2), and rotavirus (Rota). Each of the plasmids was diluted to yield control solutions with a low copy number and were quantified in a clinical laboratory by a quantitative qPCR assay used to measure viral loads in clinical specimens. The absolute copy number of target DNA in each of the solutions was further adjusted such that the copy numbers of all the plasmids were equivalent and again experimentally confirmed by the HSV qPCR assay in the clinical laboratory. Finally, the control solutions were mixed in an equimolar ratio to produce a control solution that served as a quantitative control for all the targets described above.

TABLE 1 Performance characteristics of real-time PCR assays

Target virus	Slope ± SD	R^2	Sy.x	LLOD[a]	Ref
Astrovirus	0.8124 ± 0.02649	0.9363	0.2049	1	[44]
Bovine viral diarrhea virus	0.7837 ± 0.02775	0.9257	0.2146	1	[45]
Chikungunya virus	0.7481 ± 0.02093	0.9523	0.1619	2	[46]
Encephalomyocarditis virus	0.8728 ± 0.03297	0.9175	0.2501	2	[47]
Hepatitis E virus	0.7672 ± 0.02372	0.9423	0.1835	3	[39]
Influenza A virus	0.7932 ± 0.04039	0.8635	0.2974	5	[48]
Influenza B virus	0.7724 ± 0.03054	0.9091	0.2362	1	[49]
Lymphocytic choriomeningitis virus	0.9190 ± 0.03770	0.9055	0.2821	16	[50]
Mycoplasma haemofelis group	0.7338 ± 0.03204	0.8913	0.2478	1	[18]
Mycoplasma haemominutum group	0.8471 ± 0.01765	0.973	0.1365	1	[18]
Norovirus genogroup 2	0.3875 ± 0.06981	0.325	0.54	NQ	[51]
Porcine adenovirus	0.7677 ± 0.02418	0.9403	0.187	1	[52]
Porcine cytomegalovirus	0.6950 ± 0.02204	0.9395	0.2915	1	[53]
Porcine circovirus 2	0.8727 ± 0.02690	0.9427	0.2081	3	[54]
Porcine circovirus 1	0.9100 ± 0.03768	0.9011	0.1704	5	[55]
Porcine endogenous retrovirus C	0.7965 ± 0.03060	0.9174	0.2271	1	[6]
Porcine hemagglutinating encephalomyelitis virus	0.8539 ± 0.02685	0.9405	0.2077	2	[24]
Porcine lymphotrophic herpesvirus 1	0.8478 ± 0.02887	0.9309	0.2233	2	[20]
Porcine lymphotrophic herpesvirus 2	0.8909 ± 0.02797	0.9407	0.2164	1	NA
Porcine lymphotrophic herpesvirus 3	0.9071 ± 0.01846	0.9742	0.1428	2	NA
Porcine parvovirus	0.8036 ± 0.02283	0.9509	0.1766	1	[56]
Porcine reproductive and respiratory syndrome virus	0.7535 ± 0.02709	0.9247	0.2069	2	[57]
Rabies virus	0.7906 ± 0.04070	0.8628	0.2933	4	[58]
Reovirus 1	0.8082 ± 0.03339	0.9043	0.248	9	[59]
Reovirus 2	0.7825 ± 0.03114	0.9133	0.2244	6	[59]
Reovirus 3	0.9244 ± 0.04950	0.8449	0.3829	8	[59]
Rotavirus	0.6087 ± 0.04686	0.7313	0.3507	NQ	[60]
Sapovirus	0.5695 ± 0.04044	0.756	0.3128	1	[51]
Seneca valley A virus	0.8559 ± 0.02430	0.951	0.1879	2	[61]
Transmissible gastroenteritis virus	0.8812 ± 0.02695	0.9435	0.2085	16	[22]
West Nile virus	0.4038 ± 0.04552	0.5514	0.3521	NQ	[62]

NQ, Not a quantitative assay; NA, Not applicable as it is reported here for the first time.

[a]Lower limit of detection (LLOD) is shown as copy number detected with a 95% frequency by Poisson analysis, or the concentration where fewer than 2 of 3 positive specimens were detected, whichever was greater.

2.2 | Primers and probes

Where possible, qPCR assays reported in the literature were performed as reported, while others required adaptation to be compatible with the common testing platform used to facilitate parallel evaluation of all the targets on a QuantStudio 6 Flex instrument. Target sequences for the adapted assays were designed to be within the amplified regions reported in the literature or at least overlapped with the sequences identified in the references cited in Table 1. Each of the primers and probes used in this study is shown in Table S1 together with the accession number of the nucleotide sequence used in the design of the primers and genomic coordinates for each target as well as the reference that formed the basis of the assay. All primers were obtained from a commercial supplier (Integrated DNA Technologies, Coralville IA), and probes were obtained

from the same suppliers with 5′ FAM fluorescent dye and both internal ZEN and 3′ Iowa Black quenchers. This analytical approach enables the parallel testing of all the targets at the same time and has the advantage of flexibility such that more targets can be detected by the addition of other primer and probe sets.

2.3 | Detection of infectious agents by qPCR

All steps in the setup of the amplification and detection reactions were performed on a BioMek 4000 (Beckman-Coulter, Brea, CA) using reagents arrayed in 96-well plates and a 384-well Fast PCR (Applied Biosystems) reaction plate for the qPCR. Briefly, the first reagent plate contained arrayed 5X primer and probe sets that were prepared in nuclease-free water at final concentrations of 2 μmol/L of each primer and 600 nmol/L of probe. A second reagent plate contained fast PerfeCTa qPCR ToughMix Mix (QuantaBio, Beverly, MA). The final reagent plate contained aliquots of nuclease-free water to serve as a negative control, the control plasmid mixture (see above) as a positive control for each assay, and a set of 10 cDNA samples to serve as templates (see below). Final reaction mixtures contained 2 μL of the primer/probe mix, 6 μL of the polymerase mix with reaction buffer, and 2 μL of template cDNA. The qPCR was then performed on a QuantStudio 6 with a Fast block (Applied Biosystems) using an initial 20 seconds denaturation at 95°C, and 45 cycles of 95°C 1 second, 60°C 20 seconds. Results were exported and cycle threshold (c_t) values were used to calculate the copy number using the standard formula $\log_{10}(2^{40-ct})$.

Additional controls include an internal control virus (influenza B virus, IFB) that is spiked into specimens from pigs to ensure that there were no interfering substances in the samples and that the reverse transcription and detection reactions were performing according to specifications. The internal control qPCR used to detect this control has been described previously and has been used to detect this virus in human clinical trials.[17] A control qPCR for porcine cyclophilin A sequences in genomic DNA was also included in each assay to ensure the integrity of the samples as a pre-analytic control. All the experiments were performed by methods prescribed in standard operating procedures in the laboratory to ensure the integrity of the analytical methods, and quality assurance measures confirmed the data from each experiment were valid.

2.4 | Statistical calculations

For the lower limit of detection studies, 11 2-fold dilutions of the control DNA were prepared and yielded concentrations that ranged from 10000 to 10 copies per reaction. The copy number of the diluted controls was then determined in 6 separate assays and Probit analysis was used to calculate the lowest copy number that could be detected with a frequency of 95% as a function of the variance of the experimental values. Data for the $\log_{10}$ copy number of the control plasmids and the $\log_{10}$ copy number of the experimentally determined data were entered into GraphPad Prism (La Jolla, CA) and best-fit equations, slopes, X- and Y-intercepts, R^2 coefficients, and the standard deviation of the residuals (Sy.x) were calculated. Probit

analysis of the data was performed in excel to evaluate variance in the assay over a series of different control concentrations and was used to calculate the lower limit of detection (LLOD) defined as the copy number that could be detected in 95% of the assay reactions. For qualitative assays, the LLOD was defined as the lowest copy number at which positive signals were obtained in 5 of the 6 separate determinations. In a second set of studies, a separate dilution series of 300-0.6 copies was used to confirm results of the Probit analysis with the lower limit of detection defined as the lowest concentration at which 2 of 3 concentrations were positive.

2.5 | Care of experimental animals and collection of research specimens

All studies conform to the US National Research Council's Guide for the Care and Use of Laboratory Animals, the US Public Health Service's Policy on Humane Care and Use of Laboratory Animals, and Guide for the Care and Use of Laboratory Animals. The experimental protocols used in this study were approved by the University of Alabama Birmingham Institutional Animal Care and Use Committee.

Recipient gilts were commercial Landrace x Large White crossbred animals obtained from a private/commercial farm. Gilts were approximately 7-9 months of age. The gilts were selected as normal gilts having at least two estrous recorded, before they were moved to the UAB facilities. The animals were checked for health status when they arrived from the farm. The recipient gilts received vaccinations against the following pathogens: parvovirus, *Leptospira*, *erysipelas*, swine flu, *Lawsonia*, *Mycoplasma*, circovirus, *E. coli*, *Clostridium*, and rotavirus.

The animals were located in rooms exclusively dedicated to experimental animals and were not in contact with any animal other than animals from the same experimental group. These facilities have AALAC certification and fill all the hygienic requirements for USDA research species.

Piglets were obtained by somatic cell nuclear transfer (SCNT) and were produced for research purposes. All the piglets were females, and their genetic background was Landrace x Yorkshire. Piglets were derived via caesarian section. Briefly, a 12-15 inches incision was made longitudinally in the ventromedial line, between the last teat and the umbilicus. After the abdominal cavity was accessed, the uterus was exposed, and a uterotomy incision was made along the uterine horns to remove all the fetuses. Alternatively, some litters were derived by doing a hysterectomy where the entire uterus was moved to a table and the piglets were harvested. Every piglet was extracted and handed to an assistant; the umbilical cord was clamped and dipped in 2.5% povidone-iodine, and any sign of abnormality was recorded. The newborns were then moved to a clean room dedicated to the piglets. Piglet's pens were equipped with supplemental lamps to heat the neonates. Piglets received a *Mycoplasma* + Circovirus vaccination when they were 3 weeks old.

Piglets samples were taken at birth and then at euthanasia, when the animals were 1-14 days of age. Specimens evaluated in this study typically included blood, stool swabs, and nasal swabs, but varied depending on when they were harvested. Swabs containing nasal and

stool specimens were added to standard viral transport media (VTM) supplemented with nystatin and gentamycin and mixed at a 1:1 ratio with RNAlater (Invitrogen, Carlsbad, CA). For blood specimens, material was collected in PAXgene blood RNA tubes (Thermo Fisher Scientific, Waltham, MA). All samples were frozen at −20°C prior to transfer to the laboratory for further analysis. Whole blood in PAXgene tubes was inverted to mix and stored at 4°C until shipment. Once received in the laboratory, all samples were logged in and stored at −80°C.

2.6 | Specimens testing positive for human viruses

Deidentified human specimens testing as positive for human viruses were obtained from the University of Alabama at Birmingham, Department of Pediatrics Molecular Diagnostic Laboratory. Specimen types included swabs from skin, whole blood, and plasma.

2.7 | Extraction of nucleic acids

For nasal swabs in viral transport media, DNA and RNA were extracted using an EZ1 BioRobot and the EZ1 Virus Mini Kit v2.0 (Qiagen). Briefly, lysis was performed in the presence of proteinase K and lysis buffer, which ensures digestion of viral coat proteins and inactivation of RNases. Binding buffer was added, and the lysates were mixed with magnetic particles allowing absorption of viral nucleic acids to the silica surface. Contaminants were then washed away with a sequence of wash steps. Highly purified nucleic acids were then eluted in buffer AVE and immediately stored at −80°C until cDNA could be prepared. For stool samples in VTM, the specimens were pre-treated by suspension in buffer ASL, vortexed, and incubated at room temp for 10 minutes. The specimen was then heated to 70°C for 10 minutes, and then, nucleic acids were extracted by the same methods used for nasal samples. For blood specimens, total nucleic acids were extracted using an EZ1 Virus Mini v2.0 kit and the EZ1 BioRobot. The procedure was essentially the same as for nasal specimens, but the blood specimen was suspended in buffer ATL and vortexed prior to extraction on the instrument.

2.8 | Synthesis of cDNA

All samples were reverse transcribed prior to amplification and detection of target sequences using a High Capacity RNA-to-cDNA kit (Applied Biosystems, Foster City, CA). Briefly, 2X buffer and the 20X enzyme mix were placed in a reaction tube followed by template nucleic acids and other reagents. The reaction mixture was then heated to 37°C for 60 minutes and the reaction stopped by heating to 95°C for 5 minutes. Reverse transcribed samples containing cDNA were then stored at −80°C for long-term storage.

3 | RESULTS

The overall goal of these studies was to assess specimens from sows and piglets derived by cesarean sections for infectious agents to help understand the rate of infection in these cohorts and potential transmission of viruses from sows to their piglets. Initial objectives focused on the characterization of each assay to establish reproducibility, sensitivity, and specificity as well as establish the lower limit of detection to help compare results presented here with those from other laboratories. Additional studies examined different sample types from the same animals to help understand the most efficient way to detect each of the infectious agents tested. Finally, a set of piglets derived by cesarean section were evaluated to compare their rate of infection with that from the sows from which they were derived.

3.1 | Characterization of assay performance

The adaptation of primer and probe sets reported in the literature to this common platform was achieved by increasing or decreasing the length of oligonucleotides used as primers and probes, while targeting or overlapping the same amplified region in the reports. This approach resulted in assays that were very similar in their performance characteristics and is evident in the similarity in the assay performance parameters summarized in Table 1.

Initial studies for assays for all of the agents listed in Table 1 established the LLOD by evaluating a series of control DNAs at a known copy number to assess the sensitivity of the assays. Experimental results were compared to the input copy number using statistical methods to calculate a best-fit line to assess the linearity and sensitivity of each assay. A summary of the performance of each assay is provided in Table 1 including the slope, R^2 coefficients, standard deviation of the residuals, LLOD, and a reference for each analytical assay. The data are also summarized in Figure 1 to illustrate the dynamic range, linearity, and reproducibility of each assay. Most of the assays shared similar performance characteristics, and all but 3 appeared to exhibit features of good quantitative assays.

Salient features of the assays are as follows: i) each was highly reproducible with standard deviation values that were on average ±0.2 $\log_{10}$ copies for all concentrations of the plasmids, ii) all assays were highly sensitive, iii) most yielded data that were quantitative with slopes that ranged between 0.56 and 0.91 with R^2 coefficients that ranged between 0.73 and 0.94 and corresponded to standard deviation of residual values of 0.13-0.54, iv) assays that did not prove to be quantitative were Noro, WNV, and Rota, and v) all of the quantitative assays exhibited LLOD values of between 1 and 16 copies as defined by copy number that could be detected with a frequency of 95% calculated by Probit analysis. To confirm this high level of sensitivity, a second lower set of control plasmid dilutions was prepared, and two of three assays detected cognate sequences in assays that included from 1 to 4 copies of all targets, except LCMV in which 2 of 3 specimens were detected for 10 copies (data not presented). For qualitative assays, the LLOD was defined as the lowest concentration at which 5 of 6 samples tested positive (Table 1).

The LLOD of every one of the quantitative assays was between 1 and 16 copies per assay, which is similar to the sensitivity of the validated HSV assay that is used for clinical specimens
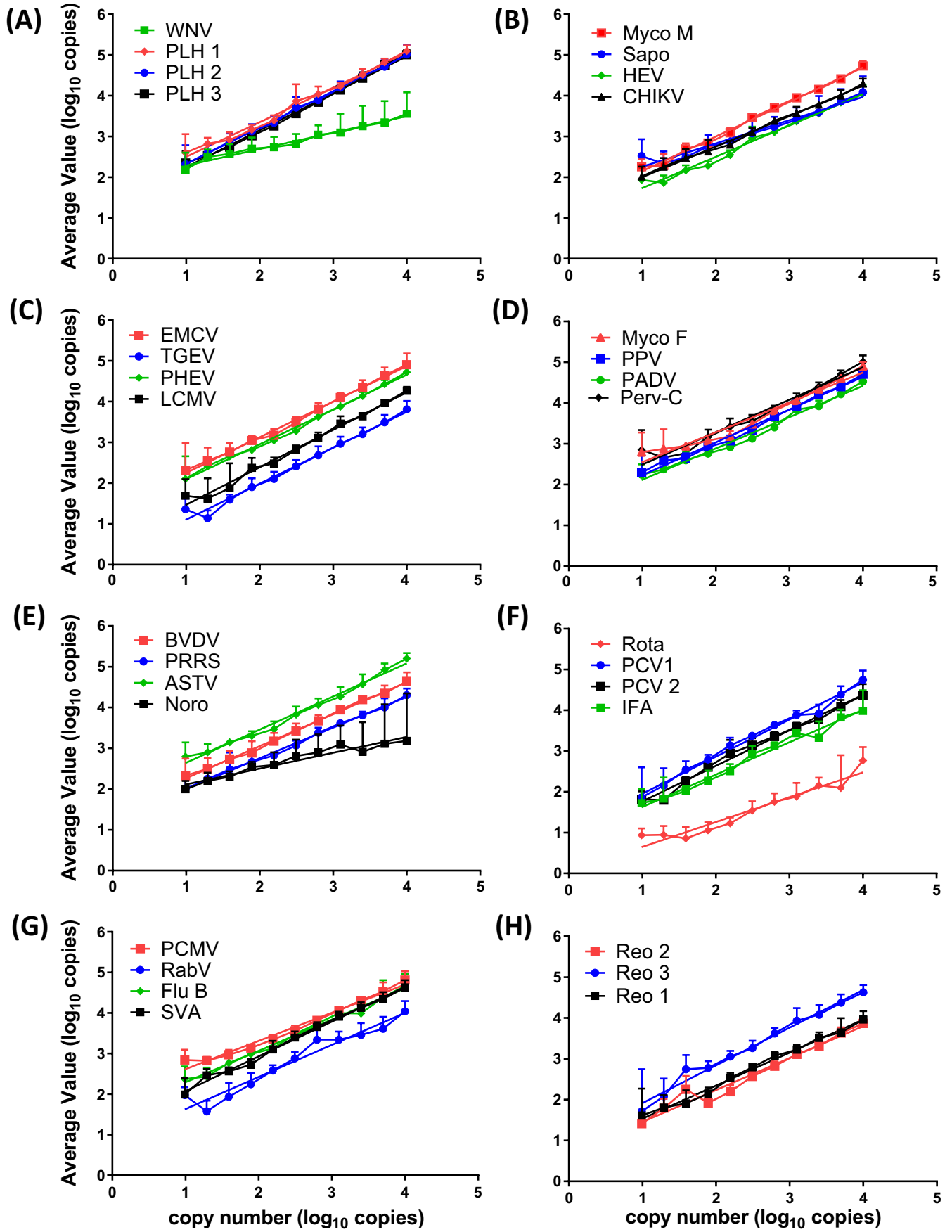
FIGURE 1 Sensitivity and linearity of qPCR assays. A set of plasmids containing each of the target nucleic acid sequences was subjected to a set of 2-fold dilutions to yield the absolute copy number shown on the horizontal axis of each XY plot. Each dilution was quantified in six separate experiments, and the average copy number was calculated and shown on the vertical axis of each plot with error bars representing the standard deviation values. Most assays yielded linear relationships with low standard deviation values and slopes approaching 1.0; however, some of the assays did not perform to a level required for quantitative assays including WNV, Noro, and Rota in panels A, E, and F, respectively. The assays for these three viruses did prove to be highly sensitive and are good qualitative assays. While most of the assays yielded similar amplification characteristics, differences in the specific amplification efficiencies can contribute to differences in both the slope and Y-intercept for some of the assays

TABLE 2 Analytical specificity specimens positive for human viruses

Source[a]	Specimen	Virus	Viral load[b]	Result[c]
Human	Blood	Epstein-Barr virus	37344	NEG
Human	Skin swab	Varicella zoster virus	3185587500	NEG
Human	Skin swab	Herpes simplex virus	36770700	NEG
Human	Blood	Human cytomegalo-virus	523	NEG
Human	Blood	Human enterovirus	Positive	NEG
Human	Blood	Human adenovirus	23252	NEG
Human	Blood	Human herpesvirus 6	154	NEG
Human	Plasma	BK polyomavirus	8210	NEG

[a]Deidentified specimens positive for human viral pathogens were obtained from the UAHSF Molecular Diagnostic Laboratory.
[b]Quantitative results are for assays using primers for the cognate viruses and are in units of copies/mL.
[c]Result for all assays in Table 1.

and was used to document the concentration of the control plasmids.[16] Further, the sensitivity of the HSV assay was also determined on this platform and was used to confirm the accuracy of LLOD estimates. The LLOD of this control assay was nearly identical to the sensitivity determined in clinical laboratory that validated the plasmid control solutions and provides assurance that the assay platform was performing as designed (data not presented). Finally, while assays for Noro, WNV, and Rota were highly sensitive, they did not appear to yield good quantitative data as indicated by the low R^2 coefficients and aberrant slope values (Table 1). Ongoing efforts in the laboratory are evaluating new primer sets with the hope of improving these assays and adding additional targets.

As expected, most of the assays (28/31) exhibited excellent linearity and yielded high-quality quantitative data (Figure 1, Table 1). Additionally, they were highly reproducible as is apparent in the error bars shown in figure 1. Three assays, WNV, Rota, and Noro, were highly sensitive but yielded low slope values and elevated standard deviations of the residual values indicating that the

assays would not generate high-quality quantitative data and thus are considered to be qualitative assays (See Figure 1A,E,F). Most importantly, most of the assays proved to be highly sensitive with the LLOD for each of the viruses of 1-16 copies per assay with most near the lower end of this range (Table 1 and Figure 1). This level of sensitivity corresponds to between 250 and 4000 copies/mL of the initial specimen. This sensitivity compares well with the sensitivity of the HSV control assay documented in the clinical laboratory (1 copy/reaction) and was similar to the performance of the HSV control assay reproduced on this assay platform. No interfering substances were detected in any of the clinical specimens evaluated in studies presented here, and the internal controls and procedures in place will ensure the consistent performance of the assay. All these data taken together support the use of this panel to provide investigators with excellent qualitative or quantitative information on each of the targets in the panel with the goal of understanding the infectious disease load in normal healthy animals and ensuring the safety of porcine tissues used in xenotransplantation research.

The analytical specificity of the assays was evaluated using a set of 8 deidentified nucleic acid specimens that tested positive for human pathogens with high viral loads where possible. Each of the specimens was positive for a single virus and included 5 different human herpesviruses (HSV, varicella-zoster virus (VZV), human cytomegalovirus (HCMV), Epstein-Barr virus (EBV), or human herpesvirus 6 (HHV6), BK polyomavirus, human adenovirus, and human enterovirus (Table 2). The only target detected in this analysis was obtained for the porcine cyclophilin A gene used to detect host DNA as a pre-analytical control for sample integrity and was expected given the high level of conservation between the pig and human genes. For this target, only three nucleotides in the 77 base pair target sequence were different between the porcine and human genes. Also, as the nucleic acids were prepared in the clinical laboratory, the samples were not spiked with influenza B virus, and as a consequence, the IFB controls were also negative as expected. Notably, none of the assays for porcine viruses detected any of the viruses in the human specimens. For instance, the primers for the porcine herpesviruses PCMV, PLHV1, PLHV2, and PLHV3 did not amplify any of the human herpesvirus sequences in the specimens including HSV, HCMV, VZV, EBV, or HHV6 even though two of the specimens tested contained more than 10^9 and 10^7 copies/ml of VZV and HSV, respectively. These data confirmed that the assays are highly specific and do not appear to cross-react with many of the infections that might be expected to occur in human subjects.

Table 3 Results for all targets on each pig specimen

Pig[a]	Type	ASTV	BVDV	CHIKV	EMCV	HEV	IFA[b]	IFB	LCMV	Myco F	Myco M	Noro	PADV	PCMV	PCV1	PCV 2
A	BLD	–	–	–	–	–	–	2.7	–	–	–	–	–	–	–	–
A	NP	–	–	–	–	–	–	4.7	–	2.0	–	–	–	–	–	–
A	STL	–	–	–	–	–	–	4.9	–	–	–	–	–	–	–	–
A1	BLD	–	–	–	–	–	–	3.5	–	–	–	–	–	–	–	–
A1	NP	–	–	–	–	–	–	4.4	–	–	–	–	–	–	–	–
A1	STL	–	–	–	–	–	–	3.3	–	–	–	–	–	–	–	–
A2	BLD	–	–	–	–	–	–	4.1	–	–	–	–	–	–	–	–
A2	NP	–	–	–	–	–	–	3.8	–	–	–	–	–	–	–	–
A2	STL	–	–	–	–	–	–	3.0	–	–	–	–	–	–	–	–
A3	BLD	–	–	–	–	–	–	3.4	–	–	–	–	–	–	–	–
A3	NP	–	–	–	–	–	–	4.3	–	–	–	–	–	–	–	–
A3	STL	–	–	–	–	–	–	3.2	–	–	–	–	–	–	–	–
A4	BLD	–	–	–	–	–	–	3.7	–	–	–	–	–	–	–	–
A5	BLD	–	–	–	–	–	–	3.6	–	–	–	–	–	–	–	–
A5	NP	–	–	–	–	–	–	4.4	–	–	–	–	–	–	–	–
A5	STL	–	–	–	–	–	–	3.7	–	–	–	–	–	–	–	–
A6	NP	–	–	–	–	–	–	4.5	–	–	–	–	–	–	–	–
A6	STL	–	–	–	–	–	–	3.7	–	–	–	–	–	–	–	–
A7	NP	–	–	–	–	–	–	3.8	–	–	–	–	–	–	–	–
A7	STL	–	–	–	–	–	–	3.2	–	–	–	–	–	–	–	–
B	NP	–	–	–	–	–	–	2.9	–	0.9	–	–	–	–	–	–
B1	NP	–	–	–	–	–	2.8[c]	2.9	–	0.5	–	–	–	–	–	–
B3	NP	–	–	–	–	–	–	2.3	–	0	–	–	–	–	–	–
B4	NP	–	–	–	–	–	–	2.6	–	–	–	–	–	–	–	–
B5	NP	–	–	–	–	–	–	2.6	–	0	–	–	–	–	–	–
B6	NP	–	–	–	–	–	–	2.1	–	–	–	–	–	–	–	–
B7	NP	–	–	–	–	–	–	2.6	–	0	–	–	–	–	–	–
C	NP	–	–	–	–	–	–	1.8	–	–	–	–	–	–	–	–
C1	NP	–	–	–	–	–	–	1.9	–	–	–	–	–	–	–	–
D	BLD	–	–	–	–	–	–	2.7	–	–	–	–	–	–	–	–
D	NP	–	–	–	–	–	–	4.0	–	–	–	–	–	–	–	–
D1	BLD	–	–	–	–	–	–	3.0	–	–	–	–	–	–	–	–
D1	NP	–	–	–	–	–	–	3.4	–	–	–	–	–	–	–	–
D2	BLD	–	–	–	–	–	–	3.0	–	–	–	–	–	–	–	–
D2	NP	–	–	–	–	–	–	4.5	–	–	–	–	–	–	–	–
D3	BLD	–	–	–	–	–	–	3.9	–	–	–	–	–	–	–	–
D3	NP	–	–	–	–	–	–	4.0	–	2.5	–	–	–	–	–	–
E	NP	–	–	–	–	–	–	2.9	–	–	–	–	–	–	–	–
E2	NP	–	–	–	–	–	–	2.3	–	0	–	–	–	–	–	–
E3	NP	–	–	–	–	–	–	2.4	–	1.0	–	–	–	–	–	–
E5	NP	–	–	–	–	–	–	2.9	–	–	–	–	–	–	–	–
E6	NP	–	–	–	–	–	–	1.5	–	–	–	–	–	–	–	–
E7	NP	–	–	–	–	–	–	1.5	–	−1	–	–	–	–	–	–
F	BLD	–	–	–	–	–	–	2.4	–	–	–	–	–	–	–	–
F	NP	–	–	–	–	–	–	4.1	–	1.7	–	–	–	–	–	–

Perv-C	PHEV	PLHV 1	PLHV 2	PLHV 3	VPP	PRRS	RabV	Reo 1	Reo 2	Reo 3	Rota	Sapo	SVA	TGEV	WNV
3.4	–	–	1.3	2.1	–	–	–	–	–	–	–	–	–	–	–
3.2	–	–	–	1.4	–	–	–	–	–	–	–	–	–	–	–
–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
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–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
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–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
–	–	–	1.7	–	–	–	–	–	–	–	–	–	–	–	–
–	–	–	–	–	–	–	–	2.0[c]	–	–	0.6[c]	–	–	–	–
–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
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–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
0	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
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–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
–	–	–	1.7	–	–	–	–	–	–	–	–	–	–	–	–
–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
0	–	–	1.5	2.8	–	–	–	–	–	–	–	–	–	–	–
–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–

(Continues)

TABLE 3 (Continued)

Pig[a]	Type	ASTV	BVDV	CHIKV	EMCV	HEV	IFA[b]	IFB	LCMV	Myco F	Myco M	Noro	PADV	PCMV	PCV1	PCV 2
F	STL	–	–	–	–	–	–	2.7	–	1.8	–	–	–	–	–	–
G	BLD	–	–	–	–	–	–	2.5	–	–	4.1	–	–	–	–	–
G	STL	–	–	–	–	–	–	3.3	–	2.4	–	–	–	–	–	–
H	BLD	–	–	–	–	–	–	2.4	–	–	–	–	–	–	–	–
H	NP	–	–	–	–	–	–	3.6	–	–	–	–	–	–	–	–
H	STL	–	–	–	–	–	–	2.3	–	1.6	–	–	–	–	–	–
I	BLD	–	–	–	–	–	–	3.2	–	–	–	–	–	–	–	–
I	NP	–	–	–	–	–	–	4.1	–	0.9	–	–	–	–	–	–
I	STL	–	–	–	–	–	–	2.3	–	1.5	–	–	–	–	–	–

ASTV, astrovirus; BVDV, bovine viral diarrhea virus; CHIKV, chikungunya virus; EMCV, encephalomyocarditis virus; HEV, hepatitis E virus; IFA, influenza A virus; IFB, influenza B virus; LCMV, lymphocytic choriomeningitis virus; Myco F, mycoplasma haemofelis group; Myco M, mycoplasma haemominutum group; Noro, norovirus genogroup 2; PADV, porcine adenovirus; PCMV, porcine cytomegalovirus; PCV1, porcine circovirus 1; PCV2, porcine circovirus 2; PERV-C, porcine endogenous retrovirus C; PHEV, porcine hemagglutinating encephalomyelitis virus; PLHV 1, porcine lymphotrophic herpesvirus 1; PLHV 2, porcine lymphotrophic herpesvirus 2; PLHV 3, porcine lymphotrophic herpesvirus 3; PPV, porcine parvovirus; PRRS, porcine reproductive and respiratory syndrome virus; RabV, rabies virus; REO1, reovirus 1; REO2, reovirus 2; REO3, reovirus 3; Rota, rotavirus; Sapo, sapovirus; SVA, Seneca valley A virus; TGEV, transmissible gastroenteritis virus; WNV, West Nile virus.

[a]Identity of 9 sows are designated by letters A to I with the subsequent number indicating the identity of the piglets derived from them.

[b]Values shown represent copy number per reaction calculated by the formula $\log10(2^{40-ct})$. Values of 0 indicate the target was detected but not quantifiable, and dashes indicate no target detected.

[c]Not reproduced after re-extraction and reanalysis.

3.2 | Evaluation of specimens from sows

The validated assays described above were used to assess blood, nasal, stool, or tissue specimens from a set of 9 adult animals and 22 piglets derived by caesarian section, and all data are presented together in Table 3. Overall, studies performed well and detected a single or small number of specific targets in some animals, while no signals were detected in assays for most of the other targets. This is consistent with the high specificity of the assays described above.

Of the specimens from adult animals, 3/9 were negative for every viral infection (without regard to PERV-C status), and two animals were negative for all viral and *mycoplasma* targets. No infections with influenza, rotavirus, parvovirus, or circovirus 2 were observed and were expected as the herd was vaccinated against these viruses and is also consistent with the data suggesting these are highly specific assays.

The most common targets detected were the haemotropic *mycoplasma* species with positive results for the *Mycoplasma haemofelis* group (MycoF) for 32% (10/31) of the animals having a positive test in at least one specimen. A single animal was positive for the *Mycoplasma haemominutum* (MycoM) group. Of interest, all the positive MycoM-positive samples from this and other studies were detected in blood specimens, while the nasal and stool specimens were generally negative. In this study, all the MycoF-positive samples were from stool or nasal specimens and were not detected in blood specimens from the same animals. These results confirm that the assays for these two groups of *mycoplasma* are highly specific and suggest that the different groups of haemotropic *mycoplasma* species have a distinct tissue tropism.[18] It is unsurprising that many of the animals were infected with *mycoplasma* species as infections with *Mycoplasma hyopneumoniae* and related species are very common infections in pigs.[19]

The gammaherpesviruses were the most frequently detected viruses in the adult animals, and both PLHV3 (5/9) and PLHV2 (3/9) were observed. Two of the animals were positive for both of these viruses. Of the 9 specimens testing positive for a gamma herpesvirus, only 2 specimens were positive for both PLHV3 and PLHV2 and indicate that the assays are specific and can distinguish between these viruses. DNA from these viruses was most often detected in blood or nasal, although one stool specimen tested positive for PLHV3 in an animal that also had a positive signal from a blood specimen. This result was largely expected as these infections are frequently detected in both feral and domestic hogs in many geographic regions.[20,21]

The assays described here can detect the coronaviruses PHEV, TGEV, and the closely related virus porcine respiratory coronavirus (PRCV), as well as porcine epidemic diarrhea virus (PEDV).[22-24] Specimens tested from the animals described above were all obtained during the summer months, and none of them tested positive for any of these viruses. However, PHEV was detected in other adult animals from specimens obtained during the winter respiratory season (data not presented) and was not unexpected as it is frequently detected in domestic animals in the US.[25]

The assay for PERV-C detected target sequences in 3/9 adult animals. Positive specimens included blood, nasal, and stool samples. In two animals, levels of PERV-C were within 1-2 orders of magnitude of the PCYP control and might indicate that the assay was detecting integrated PERV-C sequences. But in the third animal, PERV-C levels detected were very low and >4 orders of magnitude lower than the PCYP control.

Perv-C	PHEV	PLHV 1	PLHV 2	PLHV 3	VPP	PRRS	RabV	Reo 1	Reo 2	Reo 3	Rota	Sapo	SVA	TGEV	WNV
–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
4.2	–	–	–	2.6	–	–	–	–	–	–	–	–	–	–	–
2.4	–	–	–	1.4	–	–	–	–	–	–	–	–	–	–	–
–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
–	–	–	1.9	–	–	–	–	–	–	–	–	–	–	–	–
–	–	–	1.3	–	–	–	–	–	–	–	–	–	–	–	–
–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–

3.3 | Evaluation of specimens from piglets derived by caesarian section

In contrast to results with the adult animals, most of the 22 piglets delivered by caesarian section were negative for all the targets tested with the exception of a single positive nasal specimen that was positive for the *Mycoplasma haemofelis* group (Table 3). This result is consistent with results from adult animals where this target is detected most often stool and nasal specimens.

One notable result was that three separate sets of piglets were derived from sows in which PLHV2 was detected, yet none of the piglets appeared to be infected with this virus. One of the sows was also infected with PLHV3, yet did not appear to transmit either virus to any of 7 piglets delivered by caesarian section. These results confirmed that the vertical transmission of these agents is inefficient as has been confirmed by others[14,15] and also that procedures used to derive these piglets could also minimize transmission of these viruses.

A single animal (Table 3, piglet B1) yielded positive results for three separate viruses including influenza A virus, reovirus 1, and rotavirus. This result triggered an investigation as the coinfection with three viral agents was very unusual. When this specimen was re-extracted and repeated, these targets were not detected and were determined to be false positives.

Results from the testing of all 54 pig specimens indicated that the assays performed well and typically detected a small number of target nucleic acids as expected. It also showed that piglets derived by caesarian section were typically negative for targets detected in the sows from which they were derived. These data are consistent with the high specificity observed with these assays,

and typically, a single or small number of specific targets was detected, while no signals were detected in a large majority of the assays. In fact, 43 of 54 specimens were negative for each of the 27 viral targets, and all specimens from the piglets tested negative for all viral targets (except for PERV-C). Only a single sow tested negative for all targets in all specimens. No infections with influenza, rotavirus, parvovirus, or circovirus 2 were detected in the adult animals and were expected as they had been vaccinated against these viruses.

4 | DISCUSSION

The overarching goal of these studies was to provide a well-characterized set of assays for the detection of virus nucleic acids to help ensure the microbiological safety of the organs for research purposes as well as monitoring potential transmission of these agents to transplantation recipients. The list of viruses tested was determined in a separate effort that identified agents which may occur in our local geographic range at more than a nil rate with a host range extending to both humans and pigs, or that reside on the list of viruses applicable to swine in 9 CFR §113.47. The assays described hold promise as part of screening program to identify suitable donor animals, validate and release of transplantable organs for research purposes, and monitor transplant recipients; the frequency of testing and the specific specimens tested will obviously vary depending on specific needs, but the molecular assays will remain the same.

The testing approach described here is similar to the comprehensive microbiological safety approach for agarose encapsulated

porcine islet cells described by Gazda and colleagues.[26] This report utilized qPCR to detect viruses in islet cells as part of the third and fourth tier of their 4 checkpoint biosafety program for the final screening of islet cells as well as human recipients. This report also tests for Borna disease virus, porcine enterovirus, swine vesicular disease virus, pseudorabies virus, porcine teschovirus, Seoul virus, Sin Nombre virus, swine pox virus, torque tenovirus, and the alphaviruses, eastern equine encephalitis virus, Venezuelan equine encephalitis virus, and western equine encephalitis virus. These were not added to our panel on the basis geographic exclusion, lack of pathogenicity, or low prevalence. A similar qPCR screening approach was also reported by Wynyard and colleagues a part of a multilevel testing schedule for 15 viruses by culture, serological, and PCR-based methods.[27] This testing regimen included targets that were not in our panel including porcine teschovirus, pseudorabies virus, and porcine enterovirus B and were also excluded for reasons described above. Additional targets in our molecular panel included specific assays for sapovirus, astrovirus, chikungunya virus, and two groups of *mycoplasma* species.

The analytical approach presented here continues to be used to evaluate large numbers of specimens and promises to yield a large body of data on infections commonly found in herds. The epidemiological data will be valuable not only for improving the health of the animals, but also promises to help ensure the safety of tissue from animals for use in xenotransplantation studies. While some viruses detected likely represent endemic infections in the herds, others likely represent seasonal respiratory infections, or localized outbreaks of less common infections.

4.1 | *Mycoplasma* species

The most common infectious agents detected in data presented here were species in the haemotropic *mycoplasma* groups, and only 3/9 adult animals tested negative in all sample types. The assays reported previously for the two major groups of *mycoplasma* also appeared to be highly specific with some animals infected with either one or the other of the two groups of *mycoplasma*.[18] It was interesting that *mycoplasma haemominutum* group was only detected in a blood specimen, while those in the *mycoplasma haemofelis* group were detected only in nasal and stool specimens and also confirms the specificity of this assay. It was unsurprising that many of the animals were infected with *mycoplasma* species as infections with *Mycoplasma hypopneumoniae* and related species are very common infections in pigs.[19]

4.2 | Herpesviruses

The gammaherpesviruses were the most frequently detected viruses in the sows, and both PLHV3 (3/9) and PLHV2 (5/9) were observed. DNA from PLHV2 was detected in blood and nasal specimens including one animal that had a positive signal from both blood and nasal specimens. Of the 3 animals infected with PLHV3, one animal was positive in blood and nasal, a second was positive in blood,

and the third animal was positive in both blood and stool. In total, 18 specimens were tested from these animals and only two were positive for both viruses suggesting that this assay can distinguish between these viruses and is also consistent with the high specificity described for these assays in Table 2. Infections with these viruses are frequently detected in both feral and domestic hogs in many geographic regions.[20,21] These data are also consistent with human data for the gamma herpesvirus EBV where 90% of the human population worldwide carries this virus as a persistent infection, and the virus remains latent in B cells and is easily detectable by PCR even in healthy individuals.[28]

It was interesting that the sows that tested positive for PLHV2 and PLHV3 did not appear to transmit the virus to piglets derived by caesarian section. While the numbers are small, no transmission was detected from three sows infected with PLHV2 to 18 piglets derived by caesarian section. Similarly, a single sow failed to transmit PLHV3 to 7 piglets also derived by caesarian section. These data appear to suggest that procedures used to derive the piglets efficiently prevented transmission of these viruses, but it is possible that infection might have become apparent if the piglets were tested later in life.

It was somewhat surprising that the betaherpesvirus, PCMV, was not detected in any of the sows tested here, although this assay has detected this virus in subsequent specimens from adult pigs. PCMV is a common infection of pigs in many parts of the world and has been expertly reviewed recently.[29] This virus is most closely related to the human roseolaviruses, and the great majority of pigs in herds are seropositive for this virus.[30] However, infection rates in the US (12%) are reported to be much lower than those in other countries and is one possible reason why no DNA was detected.[31] Experimental primary infections with PCMV resulted in congenital infection of many of the piglets and confirmed the transplacental potential for infection with this virus.[32] This is distinct from human roseolavirus infections, with HHV-6 being transmitted congenitally in about 1% of live births without any apparent symptoms in the infants, although the integration of HHV-6 into the host genome makes the detection of congenital infections problematic.[33]

It is important to remain mindful of the potential transmission of porcine herpesviruses, such as alphaherpesvirus suid herpesvirus 1, to humans. Anecdotal reports in the literature seem to suggest that the transmission is indeed possible, yet significant barriers to human infection likely exist.[34] Potential transmission of the betaherpesvirus PCMV to human cells has also been investigated, but limitations in available reagents have thus far prevented the unequivocal documentation of transmission.[35,36] The porcine gammaherpesviruses also might have some potential to be transmitted to humans, and coinfection of cells with EBV might be a factor as the mutual transactivation of these viruses has been reported.[34] Isolation of sows during pregnancy combined with cesarean delivery and a rigorous testing regimen for the presence of virus nucleic acids in transgenic piglets should minimize the potential for transmission of this and other viruses to the piglets.

4.3 | Coronaviruses

The analytical approach described here evaluates infections with assays that can detect four coronaviruses and is important because many coronaviruses can cross species barriers readily and can cause zoonotic infections.[37] Viruses detected by assays described here include porcine hemagglutinating encephalomyelitis virus (PHEV), transmissible gastroenteritis virus (TGEV), and the closely related virus porcine respiratory coronavirus,[23] as well as porcine epidemic diarrhea virus (PEDV). None of the 9 sows described here tested positive for any coronavirus and was unsurprising as specimens were collected during the summer months when infections would be expected to be less frequent. Subsequent testing with these assays detected the betacoronavirus PHEV in specimens collected during the winter months and is consistent with reports showing that it is frequently detected in domestic animals in the US.[25] This virus is a common infection in swine in many countries including the United States and is related to the circulating human coronaviruses OC43 and HKU1.[38] Thus far, no alphacoronavirus infections have been detected including PEDV, TGEV, and porcine respiratory coronavirus.

4.4 | Other infections

The target for swine hepatitis E was not detected in any specimen notwithstanding the use of an assay reported to be highly sensitive for this virus.[39] Swine hepatitis E virus has the potential to infect humans and is described in an excellent recent review.[40] Going forward, it will be critical to monitor both sows and particularly piglets to ensure that transplanted tissue remain free of the virus.[41]

The studies presented here were limited by the availability of pig specimens that tested positive in other laboratories for each of the infectious agents described here. It is also limited by the relatively small number of animals we described in detail in this report. However, the subsequent evaluation of more than 300 additional specimens has resulted in the detection of most of the targets including SVA, Noro, IFA, REO1, PHEV, PLHV1, PLHV2, PLHV3, PRRS, Rota, PCV1, PCV2, BVDV, PCMV, PADV, MycoF, MycoM, PERV-C, P-CYP, and specimens spiked with the EMCV. The detection of these targets in pig specimens provides reassurance that the analytical methods are functioning as designed, and there is no a priori reason why some targets might be more difficult to detect than others with the methods described here. Importantly, these data and the data in Table 3 also help document the analytical specificity of each of the assays in the infectious disease panel for porcine specimens; each specimen is evaluated with 30 separate assays for different viruses in parallel so the detection of one positive target typically occurs together with negative results for the remaining 29 targets. Ideally, the assay validation process would be similar to that used for assays for human viruses that are in clinical use and have been described in reports of clinical trials.[17,42] Continued efforts to evaluate large numbers of porcine specimens is expected to yield positive samples for most of these targets and will be valuable reagents as validation studies continue.

The sensitivities of the assays described here compare favorably with the sensitivities described for other similar assays described by others.[26] For PCMV, the sensitivity of 1 copy/reaction reported here is similar to that of 2 copies/reaction reported previously,[43] and for HEV, the sensitivity of 3 copies/reaction reported here is similar to the 4-10 genomic equivalents/reaction reported previously.[39] While the sensitivities of qPCR assays used in many different laboratories are likely to be similar, the adoption of international standards by World Health Organization would help to make more direct comparisons possible in the future. Nonetheless, the high sensitivity and analytical specificity of the assays described here suggest that they perform at least as well as those described in previous reports discussed above.

The assays we describe here were selected and designed to provide the parallel evaluation of a comparatively large set of targets that were selected to help minimize the risk of disease transmission in the xenotransplantation setting. As this assay is largely automated, it is possible for an individual to evaluate at least 30 specimens per day with this panel, and the assays could also be easily multiplexed by the addition of different fluorescent labels to the probes or coded sequences to the primers. The flexibility that this approach affords will be important moving forward as information on potential risks improves, and more targets are added to the set of agents described in this work. Nonetheless, the panel reported herein promises to provide data to enhance the safety of porcine tissues for xenotransplantation and will also be valuable in the evaluation of human specimens following xenotransplantation.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.