Title: Development and validation of real-time RT-PCR assays for the detection of swine acute diarrhea syndrome coronavirus (SADS-CoV) and investigation of its presence in US swine. **NPB: #18-142 IPPA**

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Industry Summary:

There are currently six coronaviruses that can infect pigs and these include porcine epidemic diarrhea virus (PEDV), porcine deltacoronavirus (PDCoV), transmissible gastroenteritis virus (TGEV), porcine respiratory coronavirus (PRCV), porcine hemagglutinating encephalomyelitis virus (pHEV), and swine acute diarrhea syndrome coronavirus (SADS-CoV). Five of them (PEDV, PDCoV, TGEV, PRCV and pHEV) are endemic in U.S. swine. SADS-CoV has only been reported in China; its presence in the U.S. remained unknown. However, transboundary spread of infectious diseases from countries to countries via known or unknown routes including the potential spread of virus via feed and feed ingredients have occurred in recent years. Therefore, it is critical to conduct ongoing surveillance to monitor the potential emergence of SADS-CoV in U.S. pigs. In addition, PEDV, PDCoV, TGEV and SADS-CoV are all enteric pathogens and cause indistinguishable clinical signs, making differential diagnosis crucial. This study included three objectives. Objective 1: develop and validate a SADS-CoV singleplex reverse transcription real-time PCR (RT-rtPCR). Objective 2: develop and validate a PEDV/PDCoV/TGEV/SADS-CoV/XIPC 5-plex RT-rtPCR for simultaneous detection and differentiation of the four viruses (XIPC serves as an internal positive control for the PCR). Objective 3: investigate if SADS-CoV is present in the U.S. and determine the detection frequency of these swine enteric coronaviruses in U.S. swine.

Four singleplex SADS-CoV RT-rtPCR assays were evaluated and one SADS-CoV PCR was chosen for the 5-plex assay development. Some previously published singleplex PDCoV PCRs cross-reacted with sparrow deltacoronaviruses. In this study, we evaluated three singleplex PDCoV PCRs and selected one PDCoV PCR that did not cross-react with sparrow deltacoronaviruses for developing the PEDV/PDCoV/TGEV/SADS-CoV/XIPC 5-plex PCR assay.

The 5-plex PCR includes primers and probe for an exogenous internal positive control (XIPC) in addition to including primers and probes for PEDV, PDCoV, TGEV, and SADS-CoV. The internal positive control provides an additional quality assurance approach to ensure the accuracy of the PCR results. The 5-plex PCR was optimized and validated thoroughly for its analytical specificity, analytical sensitivity, and diagnostic performance. The 5-plex PCR was proved to have excellent specificity, sensitivity, and diagnostic performances.

Subsequently, the 5-plex PCR was used to determine if SADS-CoV was present in U.S. swine. Based on testing 288 clinical samples archived during 2019-2020 from diarrheic pigs in the U.S. negative for PEDV, PDCoV, TGEV, rotavirus A, B, C and several thousand clinical samples submitted to the ISU VDL during 2019-2021, there was no evidence of SADS-CoV presence in U.S. swine. Among these tested samples, 1,028 samples were found positive for at least one of PEDV,

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PDCoV and TGEV and the following detection frequency data were obtained. These included (1) 71.2% PEDV positive only, (2) 18.1% PDCoV positive only, (3) 0% TGEV positive only, (4) 10.1% PEDV and PDCoV positive, (5) 0.19% PEDV and TGEV positive, (6) 0% PDCoV and TGEV positive, and (7) 0.39% PEDV, PDCoV, and TGEV positive.

In summary, a specific and sensitive PEDV/PDCoV/TGEV/SADS-CoV/XIPC 5-plex RT-rtPCR assay was developed and thoroughly validated. This 5-plex PCR can simultaneously detect and differentiate PEDV, PDCoV, TGEV and SADS-CoV in one PCR reaction and it is an invaluable tool to determine the presence and frequency of these swine enteric coronaviruses in either single infection or co-infections. Although there is no evidence of SADS-CoV presence in the U.S. now, the availability of the PEDV/PDCoV/TGEV/SADS-CoV/XIPC 5-plex PCR will enable us to conduct ongoing surveillance and thereby we are better prepared to respond to introduction.

In addition to infecting pigs, PDCoV has been reported to cross species to infect calves, chicken embryos and chicken, turkey, and humans. SADS-CoV has also been reported for its broad cross-species tropism of infecting cell lines of various host species and primary human cells. The PEDV/PDCoV/TGEV/SADS-CoV/XIPC 5-plex PCR developed in this study should be able to not only for testing swine samples but also for testing samples from other host species if any of these CoVs are suspected in such samples.

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Key Findings:

- SADS-CoV singelplex real-time RT-PCR assays were developed and validated.
- PEDV/PDCoV/TGEV/SADS-CoV/XIPC 5-plex real-time RT-PCR was developed and thoroughly validated. It has excellent sensitivity, specificity, and diagnostic performance.
- There is no evidence of SADS-CoV presence in U.S. swine at this point.
- The PEDV/PDCoV/TGEV/SADS-CoV/XIPC 5-plex PCR is an invaluable tool to conduct ongoing surveillance to determine the presence and frequency of these swine enteric coronaviruses in either single infection or co-infections.
- Some swine coronaviruses such as PDCoV and SADS-CoV could potentially infect other host species. The PEDV/PDCoV/TGEV/SADS-CoV/XIPC 5-plex PCR should be able to not only for testing swine samples but also for testing samples from other host species if any of these CoVs are suspected in such samples.

Keywords: swine enteric coronavirus, PEDV, PDCoV, TGEV, SADS-CoV, singleplex real-time RT-PCR, 5-plex real-time RT-PCR

Scientific Abstract:

Swine enteric coronaviruses, such as porcine epidemic diarrhea virus (PEDV), porcine deltacoronavirus (PDCoV), transmissible gastroenteritis virus (TGEV), and swine acute diarrhea syndrome coronavirus (SADS-CoV), are important enteric pathogens and cause indistinguishable clinical signs, making differential diagnosis crucial. SADS-CoV had only been reported in China; its presence in the U.S. remained unknown. We aimed to develop and validate a PEDV/PDCoV/TGEV/SADS-CoV/XIPC 5-plex reverse transcription real-time PCR (RT-rtPCR) for simultaneous detection and differentiation of the four viruses (XIPC serves as an internal positive control for the PCR) and investigate the detection frequency of these viruses in U.S. swine.

Four singleplex SADS-CoV RT-rtPCR assays were evaluated for analytical specificity, analytical sensitivity, and diagnostic performance by testing 140 clinical samples in China. One SADS-CoV PCR with best performance was chosen for the 5-plex assay development. One PDCoV PCR that did not cross-react with sparrow deltacoronaviruses was developed and included for the 5-plex PCR development. The 5-plex PCR including an exogenous internal positive control (XIPC) was optimized for the concentrations of primers and probes for each target. The 5-plex PCR had excellent analytical specificity by testing against 32 different swine viral and bacterial pathogens. Based on testing serial dilutions of IVT RNAs, the limits of detection of the 5-plex PCR was 8 genomic

copies/reaction for PEDV, 4 genomic copies/reaction for PDCoV, 16 genomic copies/reaction for TGEV, and 6.8 genomic copies/reaction for SADS-CoV when Ct cut-off value was set at 37 for each virus target. Based on testing 219 clinical samples to evaluate the diagnostic performance, the 5-plex PCR had diagnostic sensitivity, specificity, and agreement of 98.96%, 95.12% and 96.80% for PEDV, 100%, 97.81% and 98.63% for PDCoV, and 100%, 100% and 100% for TGEV when compared to a commercial PEDV/TGEV/PDCoV PCR.

Then the 5-plex PCR was used to determine if SADS-CoV was present in US swine. First, 288 clinical samples archived during 2019-2020 from diarrheic pigs negative for PEDV, PDCoV, TGEV, rotavirus A, B, C were tested and all 288 samples were negative for SADS-CoV. Next, the feces, fecal swabs, and oral fluid samples submitted to the ISU VDL during Mar-Oct 2019, Nov-Dec 2020, and Feb-Apr 2021 were randomly selected for testing. All samples were negative for SADS-CoV. Among the 1,028 samples positive for at least one of PEDV, PDCoV, and TGEV, the following data were found: (1) 71.2% PEDV positive only, (2) 18.1% PDCoV positive only, (3) 0% TGEV positive only, (4) 10.1% PEDV&PDCoV positive, (5) 0.19% PEDV&TGEV positive, (6) 0% PDCoV&TGEV positive, and (7) 0.39% PEDV&PDCoV&TGEV positive.

In summary, the novel PEDV/PDCoV/TGEV/SADS-CoV/XIPC 5-plex RT-rtPCR developed and validated in this study is sensitive and specific and provides a convenient tool for detection and differentiation of these important swine enteric coronaviruses. Although there is no evidence of SADS-CoV presence in the U.S. now, the availability of the PEDV/PDCoV/TGEV/SADS-CoV/XIPC 5-plex PCR will enable us to conduct ongoing surveillance and thereby we are better prepared to respond to introduction.

Introduction:

Coronaviruses (CoVs) are enveloped, single-stranded, positive-sense RNA viruses in the order *Nidovirales* and the family *Coronaviridae*. Four genera, *Alphacoronavirus*, *Betacoronavirus*, *Gammacoronavirus*, and *Deltacoronavirus*, have been described for CoVs. In pigs, six CoVs have been identified. These include porcine epidemic diarrhea virus (PEDV), transmissible gastroenteritis virus (TGEV), porcine respiratory coronavirus (PRCV), and swine acute diarrhea syndrome coronavirus (SADS-CoV) belonging to alphacoronaviruses, porcine hemagglutinating encephalomyelitis virus (pHEV) belonging to betacoronaviruses, and porcine deltacoronavirus (PDCoV) belonging to deltacoronaviruses (Saif et al., 2019). PEDV, TGEV, PDCoV, and SADS-CoV primarily cause enteric infections in pigs. PRCV is a spike gene deletion mutant of TGEV and, in contrast to TGEV that mainly targets the enteric tract, PRCV has a predilection for the respiratory tract (Saif et al., 2019). PHEV infection ("vomiting and wasting disease") produces encephalomyelitis, rather than enteritis, and thus is not often considered when differentiating enteric infections. Therefore, the four coronaviruses (PEDV, TGEV, PDCoV, and SADS-CoV) causing indistinguishable clinical signs are often considered as swine enteric coronaviruses and differential diagnosis of them is needed.

TGEV was first described in 1946 in the U.S. (Doyle and Hutchings, 1946) and subsequently reported worldwide. Nowadays, TGEV is still endemic in the U.S. although the prevalence is low (Chen et al., 2019). PEDV was first reported in Europe in 1970s followed by detection in some Asian countries in 1980s and thereafter (Lee, 2015; Wang et al., 2016). PEDV was detected for the first time in North America in 2013 (Stevenson et al., 2013) and it is still endemic in U.S. swine. PDCoV was first reported in the feces of domestic pigs in China in 2012 (Woo et al., 2012) but its association with pig diarrhea was not reported until 2014 in the U.S. (Wang et al., 2014). SADS-CoV was first detected in China in 2017 (Gong et al., 2017; Pan et al., 2017; Zhou et al., 2018b) and, in addition to the name SADS-CoV, other names such as porcine enteric alphacoronavirus (PEAV) and swine enteric alphacoronavirus (SeACoV) have also been used to describe this virus (Gong et al., 2017; Pan et al., 2017). So far, SADS-CoV has only been reported in two provinces (Guangdong and Fujian) in China (Yang et al., 2020) and it remained unknown about the presence of SADS-CoV in U.S. swine.

Reverse transcription real-time PCR (RT-rtPCR) is a sensitive and specific assay and has been widely used for detection of many RNA viruses. TaqMan probe-based singleplex RT-rtPCR for PEDV (Madson et al., 2014; Miller et al., 2016), PDCoV (Chen et al., 2015; Chen et al., 2018; Ma et al.,

2015; Marthaler et al., 2014), TGEV (Vemulapalli et al., 2009), and SADS-CoV (Xu et al., 2019; Yang et al., 2019; Zhou et al., 2018a), duplex RT-rtPCR for PEDV and PDCoV (Zhang et al., 2016), duplex RT-rtPCR for PEDV and TGEV (Kim et al., 2007), triplex RT-rtPCR for PEDV, PDCoV and TGEV (Masuda et al., 2016), and triplex RT-rtPCR for PEDV, PDCoV and SADS-CoV (Pan et al., 2020) have been reported. Recently, a PEDV, PDCoV, TGEV, and SADS-CoV multiplex RT-rtPCR was reported by a group in China (Huang et al., 2019). However, there are concerns with some previously described PCR assays. For example, in the PEDV/TGEV duplex PCR (Kim et al., 2007) and in the PEDV/PDCoV/TGEV/SADS-CoV multiplex PCR (Huang et al., 2019), the TGEV PCR primers and probes targeting the nucleocapsid gene reacted with both TGEV and PRCV; in addition, none of these two PCRs contained an internal positive control. In 2018, novel sparrow deltacoronaviruses were identified (Chen et al., 2018) and it was then found that the previously developed membrane gene-based PDCoV PCR (Chen et al., 2015) and nucleocapsid gene-based PDCoV PCR (Chen et al., 2018) cross-reacted with sparrow deltacoronaviruses.

In this study, we developed and/or evaluated multiple singleplex RT-rtPCR assays for each swine enteric CoV and then selected one singleplex RT-rtPCR of PEDV, PDCoV, TGEV, and SADS-CoV, respectively, together with a singleplex PCR for an exogenous internal positive control (XIPC) to develop PEDV/PDCoV/TGEV/SADS-CoV/XIPC 5-plex RT-rtPCR. After thorough validation, the 5-plex PCR was used to screen large number of clinical samples collected from U.S. swine during 2019-2021 to investigate whether SADS-CoV is present in the U.S. and also to determine the detection frequency of swine enteric CoVs in U.S. swine.

Objectives:

Objective 1. Development and validation of SADS-CoV singleplex rRT-PCR assays.

Objective 2. Development and validation of PEDV/PDCoV/TGEV/SADS-CoV/XIPC 5-plex RT-rtPCR for the detection and differentiation of swine enteric coronaviruses.

Objective 3. Investigation of SADS-CoV presence by testing clinical swine samples collected in the U.S.

Materials & Methods:

Primers and probes of PCRs included in this study

Two previously published TaqMan probe-based singleplex SADS-CoV PCR assay 1 (Zhou et al., 2018a) and assay 2 (Xu et al., 2019) and two TaqMan probe-based singleplex SADS-CoV PCR assays developed in the current study (assay 3 and assay 4) were included for evaluation. The information of primers and probes is summarized in Table 1.

In order to select the appropriate singleplex PDCoV, TGEV and PEDV PCR for inclusion to develop PEDV/PDCoV/TGEV/SADS-CoV/XIPC 5-plex PCR, two previously published singleplex PDCoV PCR assay 1 (Chen et al., 2015) and assay 2 (Chen et al., 2018) and one singleplex PDCoV PCR assay developed in this study (assay 3) were evaluated. Similarly, one published TGEV nucleocapsid (N) gene-based PCR assay 1 (Huang et al., 2019), one published PEDV N gene-based PCR assay 1 (Madson et al., 2014), and one TGEV spike (S) gene-based PCR assay 2 and one PEDV N gene-based PCR assay 2 developed in this study were included for evaluation. The sequences of these PCR primers and probes are shown in Table 1.

Eventually, one singleplex PCR respectively for PEDV, PDCoV, TGEV, and SADS-CoV together with one XIPC singleplex PCR were selected to develop PEDV/PDCoV/TGEV/SADS-CoV/XIPC 5-plex PCR with information of primers and probes provided in Table 1. Primers and probe for XIPC were proprietary products developed in our laboratory and their sequences are available upon request.

A commercial PEDV/PDCoV/TGEV PCR including internal positive control Xeno (Thermo Fisher Scientific) was included in this study as a reference PCR for evaluating PEDV/PDCoV/TGEV/SADS-CoV/XIPC 5-plex PCR. However, since the commercial PEDV/PDCoV/TGEV PCR is proprietary to the company, the information of primers and probes of this assay is unavailable to us.

Viral and bacterial pathogens

One PEDV Non-S INDEL isolate USA/IN19338/2013 (Chen et al., 2014), one PEDV S INDEL isolate USA/IL20697/2014 (Chen et al., 2016), one TGEV Purdue strain (ATCC VR-763), one TGEV Miller strain (ATCC VR-1740), and one PDCoV isolate USA/IL/2014 (Chen et al., 2015) were included in this study for assay evaluation. These isolates are available at the Iowa State University Veterinary Diagnostic Laboratory (ISU VDL).

One SADS-CoV isolate GD-01/2017 (Pan et al., 2017) cultured in Vero cells available in the collaborator Dr. Yaowei Huang's laboratory in Zhejiang University, China was used to validate the singleplex SADS-CoV PCRs. The work was conducted in Dr. Huang's laboratory.

One PRCV isolate ISU 1998, one pHEV isolate (NVSL 001-PDV), and two sparrow deltacoronavirus fecal samples (17-690-7 and 17-42824) (Chen et al., 2018) available at the ISU VDL were included for assay evaluation in this study.

Other non-coronavirus viral pathogens and bacterial pathogens included in this study for evaluating assay specificity include porcine rotaviruses A, B, C, porcine circovirus 2, swine influenza A virus, porcine parainfluenza virus 1, pseudorabies virus, Senecavirus A, PRRSV-1 (Type 1), PRRSV-2 (Type 2), *E. coli*, *Salmonella typhimurium*, *Clostridium difficile*, *Clostridium perfringens*, *Brachyspira hyodysenteriae*, *Mycoplasma hyopneumoniae*, *Mycoplasma hyorhinis*, *Mycoplasma hyosynoviae*, *Actinobacillus pleuropneumonia*, *Actinobacillus suis*, *Streptococcus suis*, *Glaesserella (Haemophilus) parasuis*, *Bordetella bronchiseptica*, *Pasteurella multocida*, and *Trueperella pyogenes*. All of these pathogens are available at the ISU VDL.

Clinical samples

For evaluating the diagnostic performance of singleplex SADS-CoV PCRs, 140 fecal samples collected from Guangdong, Zhejiang, Jiangsu, Shandong, Henan, Hunan, Hebei, Jiangxi, and Anhui provinces, China from 2017-2019 were used. The testing was conducted in Dr. Huang's laboratory in China.

For evaluating the diagnostic performance of the PEDV/PDCoV/TGEV/SADS-CoV/XIPC 5-plex PCR in comparison with the reference PEDV/PDCoV/TGEV PCR, 219 clinical samples (54 fecal swabs, 53 feces, 82 oral fluids, and 30 small intestines) collected from various U.S. states and submitted to ISU VDL between 2018 and 2021 were used.

In order to investigate if SADS-CoV is present in U.S. swine, two batches of clinical samples were tested. First, 288 clinical samples, archived during 2019-2020 at the ISU VDL from diarrheic pigs in the U.S. that were negative for PEDV, PDCoV, TGEV, and porcine rotavirus A, B, C, were tested by PEDV/PDCoV/TGEV/SADS-CoV/XIPC 5-plex PCR. Next, the feces, fecal swabs, and oral fluid samples submitted to the ISU VDL during March-October 2019, November-December 2020, and February-April 2021 were randomly selected for testing by PEDV/PDCoV/TGEV/SADS-CoV/XIPC 5-plex PCR. For the 1,028 samples positive for at least one of the swine enteric coronaviruses, the detection frequency of single or co-infection of swine enteric coronaviruses was calculated.

Nucleic acid extraction

Nucleic acids were extracted from various viral and bacterial pathogens and clinical samples using a MagMAX™ Pathogen RNA/DNA kit (Thermo Fisher Scientific, Waltham, MA, USA) and a Kingfisher Flex instrument (Thermo Fisher Scientific) following the instructions of the manufacturer. One hundred microliters of the sample was used for extraction and nucleic acids were eluted into 90 µl of elution buffer. Before nucleic acid extraction, an internal positive control XIPC RNA (1 x 10^4 copies per extraction) was added to the extraction lysis buffer. Thus, the extracted nucleic acid from each sample should contain XIPC RNA in addition to the target pathogen nucleic acid.

In vitro transcribed RNA

To prepare the RNA standards for SADS-CoV, PEDV, PDCoV and TGEV, double-stranded and linear gBlock DNA fragments containing the respective CoV genomic region with T7 promoter at the upstream region was synthesized (Integrated DNA Technologies, Coralville, Iowa, USA). Specifically, the SADS-CoV gBlock DNA fragment (1165 nucleotides in length) contained the partial SDAS-CoV nucleocaspid gene, the PEDV gBlock DNA fragment (1141 nucleotides in length) contained the partial PEDV nucleocapsid gene, the PDCoV gBlock DNA fragment (1066 nucleotides in length)

contained the partial PDCoV nucleocapsid gene, and the TGEV gBlock DNA fragment (1138 nucleotides in length) contained the partial TGEV spike gene.

The gBlock DNA fragment was subjected to run-off *in vitro* transcription into RNA using a MEGAscript T7 Transcription kit (Thermo Fisher Scientific) following the manufacturer's instructions. RNA transcripts were produced, treated with DNase I and purified by MEGAclear™ Transcription Clean-Up kit (Thermo Fisher Scientific) following the manufacturer's instructions. Copy numbers of RNA transcripts were calculated based on concentrations determined by a Nanodrop 2000 spectrophotometer (Thermo Fisher Scientific). Serial dilutions of *in vitro* transcribed (IVT) RNAs were prepared using nucleic acid dilution solution (Thermo Fisher Scientific). Aliquots were frozen at -80°C for single use of each aliquot.

Limit of detection of singleplex SADS-CoV PCRs and the PEDV/PDCoV/TGEV/SADS-CoV/XIPC 5-plex PCR

Serial dilutions of SADS-CoV IVT RNA were tested by singleplex SADS-CoV PCRs to compare their analytical sensitivity.

Serial dilutions of IVT RNAs of PEDV, PDCoV, TGEV, and SADS-CoV were tested by the PEDV/PDCoV/TGEV/SADS-CoV/XIPC 5-plex PCR with 3 replicates at high concentrations and 20 replicates at low concentrations for each dilution. Then, the limit of detection (LOD) of the 5-plex PCR assay for each virus was determined.

Singleplex SADS-CoV, PDCoV, TGEV, and PEDV PCRs

For all of the singleplex SADS-CoV PCR assays 1-4, PDCoV PCR assays 1-3, TGEV PCR assays 1-2, and PEDV PCR assays 1-2, the following procedures were used. Briefly, each PCR was set up in a 20 µl reaction: 5 µl of TaqPath® 1-Step Multiplex Master Mix No ROX (Thermo Fisher Scientific), 0.3 µl of CoV forward primer at 20 µM, 0.3 µl of CoV reverse primer at 20 µM, 0.25 µl of CoV probe at 20 µM, 0.2 µl XIPC forward primer at 20 µM, 0.2 µl of XIPC reverse primer at 20 µM, 0.25 µl of XIPC probe at 20 µM, 5.5 µl nuclease-free water, and 8 µl nucleic acid extract. Amplification reactions were performed on either an ABI 7500 Fast instrument (Thermo Fisher Scientific) or an QuantStudio 5 instrument (Thermo Fisher Scientific) with the following conditions: 1 cycle of 25°C for 2 min, 1 cycle of 53°C for 10 min, 1 cycle of 95°C for 2 min, and 40 cycles of 95°C for 3 sec and 60°C for 30 sec. The analysis was done using an automatic baseline, the respective CoV detector at the threshold 0.1, and XIPC detector (Cy5) at 10% of the maximum height of the sigmoid amplification curve.

Reference (commercial) PEDV/TGEV/PDCoV PCR

A commercial VetMAX™ PEDV/TGEV/PDCoV PCR including primers and probe for Xeno internal positive control (Thermo Fisher Scientific) was included in the present study as a reference PCR (simplified as the reference PEDV/TGEV/PDCoV PCR in this report). Briefly, 6.50 µl of TaqMan® Fast 1-Step Master Mix (Thermo Fisher Scientific), 0.80 µl of Amplitaq 360 DNA Polymerase (5U/µl, Thermo Fisher Scientific), 1 µl of VetMAX PEDV/TGEV/PDCoV Primer Probe Mix (Thermo Fisher Scientific), 3.7 µl nuclease-free water, and 8 µl nucleic acid extract were included in a 20 µl reaction. Amplification reactions were performed on an ABI 7500 Fast instrument (Thermo Fisher Scientific) with the following conditions: one cycle of 50°C for 5 min, one cycle of 95°C for 10 min, and 40 cycles of 95°C for 3 sec and 60°C for 30 sec. The analysis was done using an automatic baseline, PEDV detector (LIZ equivalent to Cy5) at the threshold of 5%, TGEV detector (FAM) at the threshold of 5%, PDCoV detector (VIC) at the threshold of 5%, and Xeno detector (NED) at the threshold of 10% of the sigmoid amplification curve's maximum height, respectively. Threshold cycle (C_T) <36 was considered positive and $C_T \geq 36$ was considered negative for PEDV, TGEV and PDCoV.

PEDV/PDCoV/TGEV/SADS-CoV/XIPC 5-plex PCR

PEDV/PDCoV/TGEV/SADS-CoV/XIPC 5-plex PCR was set up using TaqPath® 1-Step Multiplex Master Mix with No ROX (Thermo Fisher Scientific). The probes for PEDV, PDCoV, TGEV, SADS-CoV, and XIPC were labeled with the fluorescence dyes JUN, ABY, VIC, FAM, and Cy5, respectively (Table 1).

The concentrations of each virus primers in the 5-plex PCR were optimized at 150 nM, 300 nM, and 900 nM and compared to the corresponding virus singleplex PCR using serially diluted virus isolates and/or IVT RNA. Eventually, the optimized 5-plex PCR was set up in a 20 µl reaction including the following components.

- 5 µl of TaqPath® 1-Step Master Mix with no ROX (Thermo Fisher Scientific);
- 0.9 µl of PEDV forward primer at 20 µM (final concentration 900 nM), 0.9 µl of PEDV reverse primer at 20 µM (final concentration 900 nM), and 0.25 µl of PEDV probe at 20 µM (final concentration 250 nM);
- 0.15 µl of PDCoV forward primer at 20 µM (final concentration 150 nM), 0.15 µl of PDCoV reverse primer at 20 µM (final concentration 150 nM), and 0.25 µl of PDCoV probe at 20 µM (final concentration 250 nM);
- 0.3 µl of TGEV forward primer at 20 µM (final concentration 300 nM), 0.3 µl of TGEV reverse primer at 20 µM (final concentration 300 nM), and 0.25 µl of TGEV probe at 20 µM (final concentration 250 nM);
- 0.3 µl of SADS-CoV forward primer at 20 µM (final concentration 300 nM), 0.3 µl of SADS-CoV reverse primer at 20 µM (final concentration 300 nM), and 0.25 µl of SADS-CoV probe at 20 µM (final concentration 250 nM);
- 0.15 µl of XIPC forward primer at 20 µM (final concentration 150 nM), 0.15 µl of XIPC reverse primer at 20 µM (final concentration 150 nM), and 0.25 µl of XIPC probe at 20 µM (final concentration 250 nM);
- 2.15 µl nuclease-free water
- 8 µl nucleic acid extract

Amplification reactions were performed on an QuantStudio 5 instrument (Thermo Fisher Scientific) with the following conditions: 1 cycle of 25°C for 2 min, 1 cycle of 53°C for 10 min, 1 cycle of 95°C for 2 min, and 40 cycles of 95°C for 3 sec and 60°C for 30 sec. The analysis was done using an automatic baseline, PEDV detector (JUN) at the threshold of 4%, PDCoV detector (ABY) at the threshold of 10%, TGEV detector (VIC) at the threshold of 5%, SADS-CoV detector (FAM) at the threshold of 5%, and XIPC detector (Cy5) at the threshold of 10% of the sigmoid amplification curve's maximum height, respectively.

Results:

Objective 1. Development and validation of SADS-CoV singleplex rRT-PCR assays.

The four SADS-CoV singleplex PCR assays 1-4 only specifically reacted with SADS-CoV IVT RNA and did not cross react with other coronaviruses (PEDV, TGEV, PDCoV, PRCV, pHEV, sparrow deltacoronavirus 17-690-7, sparrow deltacoronavirus 17-42824) and other 23 swine viral and bacterial pathogens (Table 2), indicating that all of the four evaluated SADS-CoV singleplex PCR assays had excellent analytical specificity.

Subsequently, the four SADS-CoV singleplex PCR assays were tested using serial dilutions of SADS-CoV IVT RNA. As shown in Table 3, the SADS-CoV PCR assay 1 had approximately 2 Ct higher than the assays 2, 3, and 4 at each IVT RNA dilution, suggesting that the assay 1 was less sensitive than the assays 2, 3, and 4. The SADS-CoV PCR assays 2, 3, and 4 had comparable analytical sensitivity. However, for unknown reason(s), the internal positive control XIPC failed in some reactions of the SADS-CoV PCR assay 1 and assay 4. Hence, the SADS-CoV singleplex PCR assay 2 and assay 3 were selected for further evaluations.

When serial dilutions of a SADS-CoV cell culture isolate were tested by SADS-CoV singleplex PCR assays 2 and 3, the two assays had similar detection endpoints at 10^-7 dilution (Table 4). Although two replicates of the 10^-8 dilution gave Ct values of 37.26 and 37.15 by the SADS-CoV PCR assay 3, the trend of Ct change did not make sense when compared to that of the 10^-7 dilution. Thus, the detection endpoint of the SADS-CoV PCR assay 3 was considered as 10^-7 dilution of the isolate.

The SADS-CoV singleplex PCR assays 2 and 3 were further validated using 140 swine fecal samples collected from nine provinces in China. Among the 140 clinical samples, 16 samples were

positive by both SADS-CoV singleplex PCR assays 2 and 3 with comparable Ct values for each sample between the two assays although the assay 3 gave slightly lower Ct values compared to that of the assay 2.

With all data being considered, the SADS-CoV singleplex PCR assay 3 was selected for developing PEDV/PDCoV/TGEV/SADS-CoV/XIPC 5-plex PCR.

We also evaluated a few PDCoV, TGEV, and PEDV singleplex PCR assays. As shown in Table 2, the PDCoV PCR assays 1 and 2 cross-reacted with sparrow deltacoronaviruses 17-690-7 and 17-42824, whereas the PDCoV PCR assay 3 did not cross react with these two sparrow deltacoronaviruses and only specifically detected PDCoV. Thus, the PDCoV singleplex PCR assay 3 was selected for developing PEDV/PDCoV/TGEV/SADS-CoV/XIPC 5-plex PCR. When two TGEV singleplex PCR assays were evaluated, the TGEV N gene-based PCR assay 1 cross-reacted with PRCV whereas the TGEV S gene-based PCR assay 2 did not cross-react with PRCV and only specifically detected TGEV. Hence, the TGEV singleplex PCR assay 2 was selected for developing PEDV/PDCoV/TGEV/SADS-CoV/XIPC 5-plex PCR. Both the PEDV singleplex PCR assays 1 and 2 were very specific (Table 2). However, since the amplicon size of the PEDV PCR assay 1 is 198 bp and the amplicon size of the PEDV PCR assay 2 is 75 bp (Table 1), the PEDV PCR assay 2 with smaller amplicon size was selected to develop PEDV/PDCoV/TGEV/SADS-CoV/XIPC 5-plex PCR.

Objective 2. Development and validation of PEDV/PDCoV/TGEV/SADS-CoV/XIPC 5-plex RT-rtPCR for the detection and differentiation of swine enteric coronaviruses.

Based on the data obtained in Objective 1, PEDV singleplex PCR assay 2 (probe labeled with fluorescence dye JUN), PDCoV singleplex PCR assay 3 (probe labeled with ABY), TGEV singleplex PCR assay 2 (probe labeled with VIC), SADS-CoV singleplex PCR assay 3 (probe labeled with FAM), together with XIPC internal positive control PCR (probe labeled with Cy5) were used to develop the PEDV/PDCoV/TGEV/SADS-CoV/XIPC 5-plex PCR.

As shown in Table 2, the 5-plex PCR specifically recognized PEDV, PDCoV, TGEV, and SADS-CoV with the respective fluorescence dyes and did not cross-react with other pathogens including sparrow deltacoronaviruses 17-690-7 and 17-42824. When individual PEDV, TGEV, PDCoV, and SADS-CoV or different manual mixtures of them at equal volume ratio were tested by the 5-plex PCR, all of the samples were correctly detected by the respective fluorescence dye corresponding to each virus (Table 6 and Figure 1).

Subsequently, the analytical sensitivity of PEDV/PDCoV/TGEV/SADS-CoV/XIPC 5-plex PCR was evaluated by testing RNA extracts from 10-fold serial dilutions of PEDV, TGEV, and PDCoV cell culture isolates in comparison with a reference PEDV/TGEV/PDCoV PCR. As shown in Table 7, the 5-plex PCR and the reference PEDV/TGEV/PDCoV PCR had similar detection endpoints for PEDV (10^-5 dilution) with comparable Ct values between the two assays. Similarly, the two PCR assays had equivalent detection endpoints for TGEV (10^-5 dilution) with comparable Ct values. In contrast, the 5-plex PCR assay had lower Ct values (~2 Ct differences) than the reference PCR assay in detecting PDCoV at each dilution and the 5-plex PCR had extended detection endpoint, suggesting that the 5-plex PCR had slightly higher analytical sensitivity than the reference PCR in detecting PDCoV.

The analytical sensitivity of PEDV/PDCoV/TGEV/SADS-CoV/XIPC 5-plex PCR was further evaluated by serial dilutions of *in vitro* transcribed RNAs of PEDV, PDCoV, TGEV, and SADS-CoV. At higher concentrations of IVT RNAs, three replicates per dilution were tested; at lower concentrations of IVT RNAs, 20 replicates per dilution were tested. The mean Ct value, Ct range, and the percentage of PCR-positive reactions (Ct cut-off value of 37) at each dilution are summarized in Table 8. When Ct cut-off value was set at 37, the limit of detection (at least 95% of reactions are positive) of the 5-plex PCR was 8 genomic copies/reaction for PEDV, 4 genomic copies/reaction for PDCoV, 16 genomic copies/reaction for TGEV, and 6.8 genomic copies/reaction for SADS-CoV under the conditions of this study.

The diagnostic performance of the PEDV/PDCoV/TGEV/SADS-CoV/XIPC 5-plex PCR was evaluated using 219 swine clinical samples (54 fecal swabs, 53 feces, 82 oral fluids, and 30 small intestines) collected from various U.S. states during 2018-2021 and compared to the reference PEDV/TGEV/PDCoV PCR. When the Ct cut-off value of the reference PCR was set at 36 for PEDV,

TGEV and PDCoV as established at ISU VDL, among the 219 clinical samples, 96 samples were positive for PEDV with Ct ranges of 14.5-36, 82 samples were positive for PDCoV with Ct ranges of 14.1-36, and 12 samples were positive for TGEV with Ct ranges of 14.9-31.1, by the reference PEDV/TGEV/PDCoV PCR, indicating the wide distribution of samples from strong positive to weak positive (Table 9). Compared to the reference PEDV/TGEV/PDCoV PCR, the diagnostic sensitivity, specificity, and agreement of the PEDV/PDCoV/TGEV/SADS-CoV/XIPC 5-plex PCR were 98.96%, 95.12% and 96.80% for PEDV, 100%, 97.81% and 98.63% for PDCoV, and 100%, 100% and 100% for TGEV, respectively, when cut-off C_T value of 37 was used for the 5-plex PCR analysis (Table 10).

Among the 219 clinical samples, there were 7 discrepant PEDV results, 3 discrepant PDCoV results, and 0 discrepant TGEV results between the 5-plex PCR and the reference PEDV/TGEV/PDCoV PCR (Table 10). These 10 clinical samples having discrepant results between the two PCR assays all had relatively high Ct values (Table 11). To verify the identity of virus present in these samples and to confirm the PCR results, spike gene sequencing was attempted on the 10 samples listed in Table 11. Unfortunately, due to low concentration (high C_T) of virus in these samples, sequencing was unsuccessful on these samples.

Objective 3. Investigation of SADS-CoV presence by testing clinical swine samples collected in the U.S.

In order to investigate if SADS-CoV is present in U.S. swine, 288 clinical samples archived during 2019-2020 at the ISU VDL from diarrheic pigs in the U.S. that were negative for PEDV, PDCoV, TGEV, and porcine rotavirus A, B, C, were tested by PEDV/PDCoV/TGEV/SADS-CoV/XIPC 5-plex PCR. All of the 288 clinical samples were negative for SADS-CoV, PEDV, PDCoV and TGEV by the 5-plex PCR.

Subsequently, the feces, fecal swabs, and oral fluid samples submitted to the ISU VDL during March-October 2019, November-December 2020, and February-April 2021 were randomly selected for testing by PEDV/PDCoV/TGEV/SADS-CoV/XIPC 5-plex PCR. All of the tested samples were negative for SADS-CoV. Among them, 1,028 samples were positive for at least one of PEDV, PDCoV and TGEV. These 1,028 samples were collected from at least 21 U.S. states and from pigs at different production stages (256 samples from adult, 205 samples from grower/finisher, 237 samples from nursery, 94 samples from suckling pigs, and 236 samples from pigs with unknown ages). Among the 1,028 samples, the following detection frequency data were obtained: (1) 71.2% PEDV positive only, (2) 18.1% PDCoV positive only, (3) 0% TGEV positive only, (4) 10.1% PEDV and PDCoV positive, (5) 0.19% PEDV and TGEV positive, (6) 0% PDCoV and TGEV positive, and (7) 0.39% PEDV, PDCoV, and TGEV positive.

Discussion:

Coronaviruses can infect a wide range of host species. The life-threatening severe acute respiratory syndrome (SARS), Middle East Respiratory Syndrome (MERS), and COVID-19 associated diseases in humans are caused by coronaviruses SARS-CoV-1, MERS-CoV, and SARS-CoV-2, respectively. Coronaviruses can also infect pigs. Among the six coronaviruses infecting pigs (PEDV, PDCoV, TGEV, PRCV, pHEV, and SADS-CoV), five of them (PEDV, PDCoV, TGEV, PRCV, and pHEV) are endemic in U.S. swine. Although SADS-CoV has only been reported in China, spread of transboundary diseases from countries to countries in recent years remind us of the essentiality to actively monitor the potential emergence of SADS-CoV in U.S. pigs. In addition, PEDV, PDCoV, TGEV, and SADS-CoV are all enteric pathogens causing similar clinical signs and differential diagnosis of them is needed.

In this study, we first developed and/or evaluated four SADS-CoV singleplex PCR assays. Since there are no known positive samples in the U.S. to validate SADS-CoV PCRs, we have collaborated with Dr. Yaowei Huang in China whose laboratory has access to SADS-CoV positive clinical samples and has isolated SADS-CoV in cell culture (Pan et al., 2017). Subsequently, one SADS-CoV singleplex PCR with the best performance together with selected PDCoV, TGEV, PEDV, and XIPC singleplex PCRs were used to develop a PEDV/PDCoV/TGEV/SADS-CoV/XIPC 5-plex TaqMan probe-based RT-rtPCR. Although numerous singleplex, duplex, and triplex PCRs have been developed to detect PEDV, PDCoV, TGEV, and SADS-CoV, so far there has been only one publication describing PEDV/PDCoV/TGEV/SADS-CoV multiplex RT-rtPCR (Huang et al., 2019). However, in that multiplex PCR, TGEV primers and probe targeted the nucleocapsid gene and recognized not only TGEV but also

PRCV (see Table 2 TGEV Assay 1). In contrast, in our PEDV/PDCoV/TGEV/SADS-CoV/XIPC 5-plex PCR, TGEV primers and probe were designed to target the spike gene sequence that was absent in PRCV genome; thus, our TGEV primers and probe included in the 5-plex PCR specifically recognizes TGEV and does not cross react with PRCV (Table 2). Another disadvantage of PEDV/PDCoV/TGEV/SADS-CoV multiplex PCR developed by Huang et al (2019) is that it does not include primers and probe for an internal positive control. The 5-plex PCR developed by us includes primers and probe for an exogenous internal positive control XIPC in addition to including primers and probes for PEDV, PDCoV, TGEV, and SADS-CoV. An exogenous internal positive control (XIPC) RNA is a target that is usually added to every reaction at the nucleic acid extraction step. The RT-rtPCR master mix contains primers and probe for the XIPC target, so theoretically the XIPC should amplify in every PCR reaction. The absence of amplification of the XIPC is indicative of a problem somewhere in the process, including inhibition of the RT-rtPCR reaction. If XIPC fails and the target pathogen also fails to amplify, the result for the target pathogen is inconclusive and additional testing or resubmission of the sample is recommended. Therefore, inclusion of XIPC in our 5-plex PCR provides an additional quality assurance approach to ensure the accuracy of the PCR results.

It is a challenge to develop a 5-plex PCR including 10 primers and 5 different fluorescence dye-labeled probes. After the careful design, we selected fluorescence dyes JUN, ABY, VIC, FAM, and Cy5, to respectively label the probes for PEDV, PDCoV, TGEV, SADS-CoV, and XIPC. Since the JUN dye has similar emission spectra as ROX dye (~610-617 nm), master mix containing ROX for normalization cannot be used in our 5-plex PCR. Therefore, we used TaqPath® 1-Step Multiplex Master Mix with No ROX for our 5-plex PCR. The 5-plex PCR can be run on thermal cyclers (e.g. ABI 7500 Fast instrument and QuantStudio 5) that support channels corresponding to JUN, ABY, VIC, FAM, and Cy5.

The PEDV/PDCoV/TGEV/SADS-CoV/XIPC 5-plex PCR has excellent analytical specificity. It did not react with other non-target pathogens including sparrow deltacoronaviruses (Table 2). This eliminates the concerns that some previously published PDCoV assays cross-reacted with sparrow deltacoronaviruses (Table 2). No matter PEDV, PDCoV, TGEV, and SADS-CoV is present alone or in any possible combinations in a sample, the 5-plex PCR was able to correctly detect the virus identity as demonstrated in Table 6 and Figure 1. Hence, the 5-plex PCR will be very useful to detect PEDV, PDCoV, TGEV, and SADS-CoV single infection or co-infections in clinical samples. The 5-plex PCR had comparable analytical sensitivity to the reference PEDV/TGEV/PDCoV for detecting PEDV, PDCoV and TGEV by testing serial dilutions of the cell culture isolates (Table 7). Testing serial dilutions of PEDV, PDCoV, TGEV, and SADS-CoV IVT RNAs again confirmed that the 5-plex PCR was very sensitive and had a limit of detection of 4-16 genomic copies per reaction for the four swine enteric coronaviruses. By testing 219 clinical samples with wide Ct ranges for each of PEDV, PDCoV and TGEV, it was demonstrated that the 5-plex PCR had comparable diagnostic performances (sensitivity, specificity and agreement) to the reference PEDV/TGEV/PDCoV PCR. There were 7 discrepant results for PEDV between the 5-plex PCR and the reference PCR (Table 10). In fact, the samples #23, #24, and #75 had PEDV Ct values of 36.4, 36.4, and 36.3 by the reference PCR and PEDV Ct values of 36.6, 35.1, and 35.4 by the 5-plex PCR (Table 11). If Ct cut-off value of 37 were used for both PCR assays, these three samples would have consistent PEDV results by two PCR assays. Then, only four samples (#27, #138, #144, and #158) had >3 Ct differences for PEDV by the two PCR assays. Similarly, the three samples (#113, #115, and #145) for PDCoV could have consistent results between the reference PCR and the 5-plex PCR if different Ct cut-off values were used. In this study, Ct cut-off value of 37 was tentatively used for the 5-plex PCR. Ct cut-off value of 36 was used at ISU VDL for the reference PEDV/TGEV/PDCoV PCR (Thermo Fisher Scientific). Further optimization of the Ct cut-off values of these two PCR assays could potentially increase the equivalent results of the two PCR assays.

To investigate whether SADS-CoV is present in U.S. swine, we run the 5-plex PCR on 288 clinical samples collected from diarrheic pigs during 2019-2020 in the U.S. that were negative for PEDV, PDCoV, TGEV, and porcine rotavirus A, B, C. We thought these pigs with diarrhea but negative for the common swine enteric viruses could be a good start to test the presence of SADS-CoV in U.S. pigs. It turned out that all of these 288 pig samples were negative for SADS-CoV. Subsequently, we tested several thousand of fecal swab, feces and oral fluid samples randomly selected from the samples submitted to the ISU VDL from 2019-2021 by the 5-plex PCR. Again, all of the tested samples were negative for SADS-CoV. Interestingly, 1,028 samples were found positive for at least one of PEDV,

PDCoV and TGEV. We were able to calculate the frequency of PEDV, PDCoV, and TGEV single infection or co-infection in these 1,028 samples, providing some baseline data to understand the epidemiology of swine enteric coronaviruses in U.S. swine.

In summary, a specific and sensitive PEDV/PDCoV/TGEV/SADS-CoV/XIPC 5-plex RT-rtPCR assay was developed and thoroughly validated. This 5-plex PCR can simultaneously detect and differentiate PEDV, PDCoV, TGEV and SADS-CoV in one PCR reaction. Although our data in the current study indicate that there was no evidence of SADS-CoV presence in the U.S. now, the availability of the PEDV/PDCoV/TGEV/SADS-CoV/XIPC 5-plex PCR will enable us to conduct ongoing surveillance and thereby we are better prepared to respond to introduction.

In addition to infecting pigs, PDCoV has been reported to cross species to infect calves (Jung et al., 2017), chicken embryos and chicken (Liang et al., 2019), and turkeys (Boley et al., 2020). One recent preprint article also reported that PDCoV could infect children (Lednicky et al., 2021), raising the public health concern of PDCoV. SADS-CoV also has broad cross-species tropism of infecting cell lines from bats, mice, rats, gerbils, hamsters, pigs, chickens, nonhuman primates, and humans (Yang et al., 2019). A recent paper reported that SADS-CoV replicates in primary human cells, concerning SADS-CoV as a potential zoonotic pathogen (Edwards et al., 2020). The PEDV/PDCoV/TGEV/SADS-CoV/XIPC 5-plex PCR developed by us should be able to not only for testing swine samples but also for testing samples from other host species if any of these CoVs are suspected in such samples.

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Table 1. Sequences of primers and probes used in this study

Assay	Primer/Probe name	Sequence (5'-3')	Target gene	Amplicon size	Reference
Singleplex SADS-CoV PCR					
Assay 1	SADS-N-F1	CTGACTGTTGTTGAGGTTAC	N	155 bp	Zhou L et al, J Virol Methods. 2018, 255:66-70
	SADS-N-R1	TCTGCCAAAGCTTGTTTAAC	N		
	SADS-N-Prb1	FAM/TCACAGTCT/ZEN/CGTTCTCGCAATCA/3IABkFQ	N		
Assay 2	SADS-N-F2	GCACTTTTATTACCTTGGTA	N	144 bp	Xu et al, Transbound Emerg Dis. 2019, 66:119-130
	SADS-N-R2	GTAGCAGGTTCTTTGTTAC	N		
	SADS-N-Prb2	FAM/TCCTCACGC/ZEN/AGATGCTCCTT/3IABkFQ	N		
Assay 3	SADS-N-F3	CCAGGCCTCAAAGTGGTAAAAA	N	85 bp	This study
	SADS-N-R3	TGCTTACGAGCCGGTTTAGG	N		
	SADS-N-Prb3	FAM/ACCCAAACC/ZEN/AAGAAGCAGAGCTGTCTCAC/3IABkFQ	N		
Assay 4	SADS-N-F4	TTGGCAGACTTGGGCATAGC	N	85 bp	This study
	SADS-N-R4	GTGAGACAGCTCTGCTTCTTGGT	N		
	SADS-N-Prb4	FAM/TCCAGGCCT/ZEN/CAAAGTGGTAAAAATACACCC/3IABkFQ	N		
Singleplex PDCoV PCR					
Assay 1	PDCoV-M-RTF	CGACCACATGGCTCCAATTC	M	70 bp	Chen et al, Virology. 2015, 482:51-59
	PDCoV-M-RTR	CAGCTCTTGCCCATGTAGCTT	M		
	PDCoV-M-RTP	FAM/CACACCAGT/ZEN/CGTTAAGCATGGCAAGC/3IABkFQ	M		
Assay 2	PDCoV-N-RTF	CCTACTACTGACGCGTCTTGGTT	N	75 bp	Chen et al, Emerg Microbes Infect. 2018, 7:105
	PDCoV-N-RTR	TGCCACGAAACTGAGGATGA	N		
	PDCoV-N-RTP	VIC/TGCTCAAAGCTCAAAAC/MGB	N		
Assay 3	PDCoV-N-F2	CCAGACATGTGCCTGGTGTT	N	68 bp	This study
	PDCoV-N-R2	CCCYGCCTGAAAGTTGCT	N		
	PDCoV-N-Prb2	ABY/ARATGCTTTTCGCTGGCCACCTTG/QSY	N		

(cont. Table 1)

Singleplex TGEV PCR

	TGEV-N-F	TGCCATGAACAAACCAAC	N		Huang et al, Appl Microbiol
Assay 1	TGEV-N-R	GGCACTTTACCATCGAAT	N	81 bp	Biotechnol. 2019, 103:4943-
	TGEV-N-Prb	VIC/TAGCACCACGACTACCAAGC/MGB-3’	N		4952
	TGEV-S-F2	GTGGTAATATGYTRTATGGCYTACAA	S		
Assay 2	TGEV-S-R2	GCCAGACCATTGATTTTCAAAACT	S	101 bp	This study
	TGEV-S-Prb2	VIC/TTGCTTATTTACATGGTGCYAGT/MGB	S		
Singleplex PEDV PCR					
	PEDV-N306-F	CGCAAAGACTGAACCCACTAACCT	N		Madson et al, Vet Microbiol.
Assay 1	PEDV-N503-R	TTGCCTCTGTTGTTACTTGGAGAT	N	198 bp	2014, 174:60-68
	PEDV-N469-Prb	FAM/TGTTGCCATTACCACGACTCCTGC/QSY	N		
	PEDV-N1195-F	GAAGAGGCCATCTACGATGATGT	N		
Assay 2	PEDV-N1269-R	AACAGCTGTGTCCCATTCCAA	N	75 bp	This study
	PEDV-N1221-Prb	JUN/TGTGCCATCTGATGTGACTCATGCCA/QSY	N		
PEDV/PDCoV/TGEV/SADS-CoV/XIPC 5-plex PCR					
	PEDV-N1195-F	GAAGAGGCCATCTACGATGATGT	N		
	PEDV-N1269-R	AACAGCTGTGTCCCATTCCAA	N	75 bp	
	PEDV-N1221-Prb	**JUN**/TGTGCCATCTGATGTGACTCATGCCA/**QSY**	N		
	PDCoV-N-F2	CCAGACATGTGCCTGGTGTT	N		
	PDCoV-N-R2	CCCYGCCTGAAAGTTGCT	N	68 bp	
	PDCoV-N-Prb2	**ABY**/ARATGCTTTTCGCTGGCCACCTTG/**QSY**	N		This study
	TGEV-S-F2	GTGGTAATATGYTRTATGGCYTACAA	S		
	TGEV-S-R2	GCCAGACCATTGATTTTCAAAACT	S	101 bp	
	TGEV-S-Prb2	**VIC**/TTGCTTATTTACATGGTGCYAGT/**MGB**	S		
	SADS-N-F3	CCAGGCCTCAAAGTGGTAAAAA	N		
	SADS-N-R3	TGCTTACGAGCCGGTTTAGG	N	85 bp	
	SADS-N-Prb3b	**FAM**/ACCCAAACCAAGAAGCAGAGCTGTCTCAC/**QSY**	N		

Table 2. Analytical specificity of various singleplex and multiplex PCRs evaluated in this study

Pathogens	Singleplex PCR											PEDV/PDCoV/ TGEV/SADS-CoV/ XIPC 5-plex PCR	Reference PEDV/TGEV/ PDCoV PCR
	SADS-CoV Assay 1	SADS-CoV Assay 2	SADS-CoV Assay 3	SADS-CoV Assay 4	PDCoV Assay 1	PDCoV Assay 2	PDCoV Assay 3	TGEV Assay 1	TGEV Assay 2	PEDV Assay 1	PEDV Assay 2		
Porcine epidemic diarrhea virus (PEDV)	≥40	≥40	≥40	≥40	≥40	≥40	≥40	≥40	≥40	18.21	17.05	16.09	14.51
Transmissible gastroenteritis virus (TGEV) Purdue strain	≥40	≥40	≥40	≥40	≥40	≥40	≥40	15.00	17.43	≥40	≥40	15.87	15.41
Transmissible gastroenteritis virus (TGEV) Miller strain	≥40	≥40	≥40	≥40	≥40	≥40	≥40	17.23	19.51	≥40	≥40	17.53	16.93
Porcine deltacoronavirus (PDCoV)	≥40	≥40	≥40	≥40	17.93	17.45	14.01	≥40	≥40	≥40	≥40	16.52	17.35
SADS-CoV N-gene *in-vitro* transcribed RNA	28.35	26.05	25.85	25.15	≥40	≥40	≥40	≥40	≥40	≥40	≥40	27.56	≥40
Porcine respiratory coronavirus	≥40	≥40	≥40	≥40	≥40	≥40	≥40	18.04	≥40	≥40	≥40	≥40	≥40
Porcine hemagglutinating encephalomyelitis virus	≥40	≥40	≥40	≥40	≥40	≥40	≥40	≥40	≥40	≥40	≥40	≥40	≥40
Sparrow DCoV (17-690-7)	≥40	≥40	≥40	≥40	34.72	30.91	≥40	≥40	≥40	≥40	≥40	≥40	≥40
Sparrow DCoV (17-42824)	≥40	≥40	≥40	≥40	33.08	27.30	≥40	≥40	≥40	≥40	≥40	≥40	≥40
Porcine rotavirus (A, B, C)	≥40	≥40	≥40	≥40	≥40	≥40	≥40	≥40	≥40	≥40	≥40	≥40	≥40
Porcine circovirus 2	≥40	≥40	≥40	≥40	≥40	≥40	≥40	≥40	≥40	≥40	≥40	≥40	≥40
Influenza A virus	≥40	≥40	≥40	≥40	≥40	≥40	≥40	≥40	≥40	≥40	≥40	≥40	≥40
Porcine parainfluenza virus type	≥40	≥40	≥40	≥40	≥40	≥40	≥40	≥40	≥40	≥40	≥40	≥40	≥40
Pseudo rabies virus	≥40	≥40	≥40	≥40	≥40	≥40	≥40	≥40	≥40	≥40	≥40	≥40	≥40
Senecavirus A	≥40	≥40	≥40	≥40	≥40	≥40	≥40	≥40	≥40	≥40	≥40	≥40	≥40
PRRSV-1 (EU) Lelystad	≥40	≥40	≥40	≥40	≥40	≥40	≥40	≥40	≥40	≥40	≥40	≥40	≥40
PRRSV-2 (NA) VR-2385	≥40	≥40	≥40	≥40	≥40	≥40	≥40	≥40	≥40	≥40	≥40	≥40	≥40
Escherichia coli	≥40	≥40	≥40	≥40	≥40	≥40	≥40	≥40	≥40	≥40	≥40	≥40	≥40
Salmonella typhimurium	≥40	≥40	≥40	≥40	≥40	≥40	≥40	≥40	≥40	≥40	≥40	≥40	≥40
Clostridium difficule	≥40	≥40	≥40	≥40	≥40	≥40	≥40	≥40	≥40	≥40	≥40	≥40	≥40
Clostridium perfingens	≥40	≥40	≥40	≥40	≥40	≥40	≥40	≥40	≥40	≥40	≥40	≥40	≥40
Brachyspira hyodysenteriae	≥40	≥40	≥40	≥40	≥40	≥40	≥40	≥40	≥40	≥40	≥40	≥40	≥40
Mycoplasma hyopneumoniae	≥40	≥40	≥40	≥40	≥40	≥40	≥40	≥40	≥40	≥40	≥40	≥40	≥40
Mycoplasma hyorhinis	≥40	≥40	≥40	≥40	≥40	≥40	≥40	≥40	≥40	≥40	≥40	≥40	≥40
Mycoplasma hyosynoviae	≥40	≥40	≥40	≥40	≥40	≥40	≥40	≥40	≥40	≥40	≥40	≥40	≥40
Actinobacillus pleuropneumoniae	≥40	≥40	≥40	≥40	≥40	≥40	≥40	≥40	≥40	≥40	≥40	≥40	≥40
Actinobacillus suis	≥40	≥40	≥40	≥40	≥40	≥40	≥40	≥40	≥40	≥40	≥40	≥40	≥40
Streptococcus suis	≥40	≥40	≥40	≥40	≥40	≥40	≥40	≥40	≥40	≥40	≥40	≥40	≥40
Glaesserella parasuis	≥40	≥40	≥40	≥40	≥40	≥40	≥40	≥40	≥40	≥40	≥40	≥40	≥40
Bordetella bronchiseptica	≥40	≥40	≥40	≥40	≥40	≥40	≥40	≥40	≥40	≥40	≥40	≥40	≥40
Pasturella multocida	≥40	≥40	≥40	≥40	≥40	≥40	≥40	≥40	≥40	≥40	≥40	≥40	≥40
Trueperella pyogenes	≥40	≥40	≥40	≥40	≥40	≥40	≥40	≥40	≥40	≥40	≥40	≥40	≥40

Table 3. Analytical sensitivity of 4 SADS-CoV singleplex RT-rtPCRs by testing serial dilutions of in vitro transcribed RNA

SADS-CoV IVT RNA	SADS-CoV PCR Assay 1		SADS-CoV PCR Assay 2		SADS-CoV PCR Assay 3		SADS-CoV PCR Assay 4	
	SADS-CoV Ct	XIPC Ct	SADS-CoV Ct	XIPC Ct	SADS-CoV Ct	XIPC Ct	SADS-CoV Ct	XIPC Ct
5 x 10^7 copies/rxn	12.1574	38.4190	10.7109	33.6775	10.5395	34.8011	9.5279	≥40; Fail
5 x 10^7 copies/rxn	12.2578	≥40; Fail	10.6327	33.8087	10.6402	34.8776	9.9971	≥40; Fail
5 x 10^7 copies/rxn	12.2556	≥40; Fail	10.7148	33.6626	10.2404	35.0007	9.9523	≥40; Fail
5 x 10^6 copies/rxn	15.9878	36.7664	14.1894	33.5617	14.5550	34.6095	13.4017	≥40; Fail
5 x 10^6 copies/rxn	16.2177	38.3896	14.3726	33.6799	14.4822	34.6935	13.5329	≥40; Fail
5 x 10^6 copies/rxn	16.4319	39.9451	14.3936	33.7733	14.4082	34.8588	13.8244	≥40; Fail
5 x 10^5 copies/rxn	20.2109	34.6714	18.4316	33.5100	18.4174	34.4757	17.6620	≥40; Fail
5 x 10^5 copies/rxn	20.5274	35.5944	18.4146	33.4457	18.4753	34.5988	17.6019	≥40; Fail
5 x 10^5 copies/rxn	20.5131	36.8697	18.4666	33.7506	18.4435	34.7003	17.9031	≥40; Fail
5 x 10^4 copies/rxn	23.5501	33.9468	21.8546	33.3426	21.8894	34.2611	21.0322	≥40; Fail
5 x 10^4 copies/rxn	23.9134	34.1781	22.0473	33.5831	22.0006	34.3680	21.1146	≥40; Fail
5 x 10^4 copies/rxn	24.0189	34.3263	21.9809	33.4805	21.9783	34.3419	21.1569	≥40; Fail
5 x 10^3 copies/rxn	28.0169	33.5640	25.9694	33.5924	25.7023	34.2115	25.1418	36.4704
5 x 10^3 copies/rxn	28.0286	33.3130	26.0361	33.9181	25.7344	34.3704	25.0813	37.4585
5 x 10^3 copies/rxn	28.3509	33.4022	26.0464	33.5552	25.8554	34.2269	25.1531	38.2361
5 x 10^2 copies/rxn	32.3171	33.3842	30.6330	33.6224	29.2764	34.1100	29.6052	34.1242
5 x 10^2 copies/rxn	32.3636	33.5574	30.1855	33.6988	29.9172	34.2739	29.5876	34.2965
5 x 10^2 copies/rxn	32.8001	33.3287	30.3363	33.7369	30.0794	34.2039	29.7558	34.2790
5 x 10^1 copies/rxn	35.7512	33.3976	35.1469	33.6142	33.5564	34.3054	33.3345	33.9454
5 x 10^1 copies/rxn	35.1821	33.6067	33.8632	34.0134	33.2225	34.3225	32.6212	34.2632
5 x 10^1 copies/rxn	35.7674	33.6636	34.2610	33.9433	33.5556	34.2102	33.0614	34.3202
5 copies/rxn	≥40	33.4446	≥40	33.6495	37.3266	33.9369	≥40	34.1706
5 copies/rxn	≥40	33.3734	≥40	33.6416	37.1378	34.3292	38.8318	34.1084
5 copies/rxn	≥40	33.4383	≥40	33.6524	38.5014	34.4413	38.9690	34.1027
5 copies/rxn	≥40	33.4712	≥40	33.7581	36.2244	34.3305	36.2717	34.0158
5 copies/rxn	≥40	33.4222	≥40	33.6438	38.7615	34.1693	38.1850	33.9857
0.5 copies/rxn	≥40	33.2860	≥40	33.7005	≥40	33.9255	≥40	33.9657
0.5 copies/rxn	≥40	33.4267	≥40	33.7387	≥40	34.0511	≥40	34.1396
0.5 copies/rxn	≥40	33.4229	≥40	33.7078	≥40	34.0890	≥40	33.9334
0.5 copies/rxn	≥40	33.1993	≥40	33.8903	≥40	34.4019	≥40	33.9818
0.5 copies/rxn	≥40	33.4488	≥40	33.8425	≥40	34.2409	≥40	34.0952

Note: XIPC is internal positive control

Table 4. Analytical sensitivity of Singleplex SADS-CoV PCR assays 2 and 3 by testing serial dilutions of a SADS-CoV cell culture isolate*

Dilution	**Theoretical titer (TCID50/mL)**	**SADS-CoV Assay 2**			**SADS-CoV Assay 3**		
		Result 1	Result 2	Result 3	Result 1	Result 2	Result 3
10^-2	5 x 10^5	20.64	20.68	20.69	20.76	20.76	20.69
10^-3	5 x 10^4	24.05	24.03	24.03	24.01	23.97	24.13
10^-4	5 x 10^3	27.51	27.47	27.59	27.44	27.36	27.42
10^-5	5 x 10^2	31.00	30.99	31.23	31.10	31.06	31.24
10^-6	5 x 10^1	34.37	34.25	33.98	34.78	34.10	34.95
10^-7	5 x 10^0	38.45	37.24	38.23	37.45	37.52	36.58
10^-8	5 x 10^-1	≥40	≥40	≥40	37.26	37.15	≥40
10^-9	5 x 10^-2	≥40	≥40	≥40	≥40	≥40	≥40

* The work was conducted in a collaborator's lab in China

Table 5. Diagnostic performance of singleplex SADS-CoV PCR assays 2 and 3 by testing clinical samples

Sample ID	Specimen	SADS-CoV PCR Ct value	
		Assay 2	Assay 3
#1	Fecal	31.18	30.14
#2	Fecal	33.06	31.8
#3	Fecal	31.77	30.81
#4	Fecal	32.83	31.69
#5	Fecal	35.19	34.45
#6	Fecal	32.78	31.83
#7	Fecal	≥40	≥40
#8	Fecal	≥40	≥40
#9	Fecal	30.15	29.14
#10	Fecal	31.65	29.16
#11	Fecal	32.23	31.43
#12	Fecal	32.99	32.28
#13	Fecal	18.22	17.97
#14	Fecal	28.12	27.2
#15	Fecal	≥40	≥40
#16	Fecal	≥40	≥40
#17	Fecal	35.16	34.26
#18	Fecal	30.08	29.03
#19	Fecal	32.53	30.86
#20	Fecal	28.57	27.67
#21	Fecal	≥40	≥40
#22	Fecal	≥40	≥40
#23	Fecal	≥40	≥40
#24	Fecal	≥40	≥40
#25 - #140	Fecal	≥40	≥40

Table 6. Different mixtures of PEDV, TGEV, PDCoV and SADS-CoV tested by the PEDV/PDCoV/TGEV/SADS-CoV/XIPC 5-plex PCR

Sample No.	Sample detail (volume mixing ratio)	PEDV/PDCoV/TGEV/SADS-CoV/XIPC 5-plex PCR Ct value				
		PEDV (JUN)	TGEV (VIC)	PDCoV (ABY)	SADS-CoV (FAM)	XIPC (Cy5)
Sample #1	PEDV USA/IN19338/2013	16.744	≥40	≥40	≥40	30.369
Sample #2	TGEV Purdue (VR-763)	≥40	15.197	≥40	≥40	30.736
Sample #3	PDCoV USA/IL/2014	≥40	≥40	16.488	≥40	30.996
Sample #4	SADS-CoV N-gene IVT RNA (10^-4 dilution)	≥40	≥40	≥40	27.174	25.111
Sample #5	PEDV & TGEV (1:1)	18.749	17.684	≥40	≥40	30.037
Sample #6	PEDV & PDCoV (1:1)	18.843	≥40	17.274	≥40	31.139
Sample #7	TGEV & PDCoV (1:1)	≥40	17.329	17.771	≥40	31.484
Sample #8	PEDV & SADS-CoV (1:1)	17.239	≥40	≥40	27.974	31.061
Sample #9	TGEV & SADS-CoV (1:1)	≥40	16.314	≥40	27.991	31.596
Sample #10	PDCoV & SADS-CoV (1:1)	≥40	≥40	16.877	28.325	32.032
Sample #11	PEDV & TGEV & PDCoV (1:1:1)	20.338	17.386	17.14	≥40	32.158
Sample #12	PEDV & TGEV & SADS-CoV (1:1:1)	19.167	18.269	≥40	28.261	30.883
Sample #13	PEDV & PDCoV & SADS-CoV (1:1:1)	18.961	≥40	17.151	28.857	31.689
Sample #14	TGEV & PDCoV & SADS-CoV (1:1:1)	≥40	17.708	18.202	28.627	31.692
Sample #15	PEDV & TGEV & PDCoV & SADS-CoV (1:1:1:1)	20.684	18.009	17.806	29.673	33.799
Sample #16	Nuclease-free water without XIPC	≥40	≥40	≥40	≥40	≥40

Notes:

1. For samples #5 - #15, the respective viruses were manually mixed at the equal volume ratio.
2. PEDV probe was labeled with fluorescence dye JUN
3. TGEV probe was labeled with fluorescence dye VIC
4. PDCoV probe was labeled with fluorescence dye ABY
5. SADS-CoV probe was labeled with fluorescence dye FAM
6. XIPC is an internal positive control and its probe was labeled with fluorescence dye CY5

Table 7. Analytical sensitivity of PEDV/PDCoV/TGEV/SADS-CoV/XIPC 5-plex PCR and the reference PEDV/TGEV/PDCoV PCR by testing serial dilutions of virus isolates

Dilution	PEDV Non-S INDEL isolate USA/IN19338/2013		PEDV S INDEL isolate USA/IL20697/2014		TGEV Purdue isolate (VR-763)		TGEV Miller isolate (VR-1740)		PDCoV isolate (USA/IL/2014)	
	5-plex PCR Ct value	Reference PCR Ct value	5-plex PCR Ct value	Reference PCR Ct value	5-plex PCR Ct value	Reference PCR Ct value	5-plex PCR Ct value	Reference PCR Ct value	5-plex PCR Ct value	Reference PCR Ct value
10^-1	21.38	22.03	21.22	21.55	19.72	20.07	21.27	21.09	20.5	22.5
10^-2	25.58	25.7	24.97	25.32	23.59	23.58	25.34	25.06	24.59	26.77
10^-3	28.76	29.42	28.51	28.94	27.71	27.43	30.59	30.19	28.02	30.52
10^-4	33.17	33.14	31.6	32.36	31.7	31.65	34.06	34.05	31.26	33.6
10^-5	36.76	37.08	35.47	36.07	35.95	36.02	38.16	38.02	34.38	37.97
10^-6	≥40	≥40	≥40	≥40	≥40	≥40	≥40	≥40	36.13	39.7
10^-7	≥40	≥40	≥40	≥40	≥40	≥40	≥40	≥40	≥40	≥40
10^-8	≥40	≥40	≥40	≥40	≥40	≥40	≥40	≥40	≥40	≥40

Note: The reference PEDV/TGEV/PDCoV PCR is the commercial VETMAX PEDV/TGEV/PDCoV PCR.

Table 8. Limit of detection of PEDV/PDCoV/TGEV/SADS-CoV/XIPC 5-plex PCR by testing serial dilutions of in vitro transcribed RNA

Genomic copies per reaction	PEDV/PDCoV/TGEV/SADS-CoV/XIPC 5-plex PCR[*]				
	% (No. of Pos for PEDV)	% (No. of Pos for PDCoV)	% (No. of Pos for TGEV)	% (No. of Pos for SADS-CoV)	Mean Ct (range)
PEDV N-gene IVT RNA					
8 x 10^7	100% (3/3)				10.69 (10.64-10.77)
8 x 10^6	100% (3/3)				13.94 (13.86-14.03)
8 x 10^5	100% (3/3)				17.37 (17.32-17.41)
8 x 10^4	100% (3/3)				20.73 (20.72-20.75)
8 x 10^3	100% (3/3)				24.28 (24.24-24.33)
8 x 10^2	100% (3/3)				27.71 (27.63-27.79)
8 x 10^1	100% (20/20)				31.35 (30.85-32.26)
16	100% (20/20)				33.76 (32.72-35.04)
8	100% (20/20)				34.55 (33.36-35.80)
4	80% (16/20)				35.7 (34.31-38.05)
2	45% (9/20)				37.53 (35.27-40)
1	5% (1/20)				38.70 (36.85-40)
PDCoV N-gene IVT RNA					
8 x 10^7		100% (3/3)			11.07 (11.02-11.14)
8 x 10^6		100% (3/3)			14.02 (14.01-14.02)
8 x 10^5		100% (3/3)			17.41 (17.35-17.48)
8 x 10^4		100% (3/3)			21.29 (21.23-21.35)
8 x 10^3		100% (3/3)			24.02 (23.96-24.11)
8 x 10^2		100% (3/3)			27.64 (27.56-27.72)
8 x 10^1		100% (3/3)			31.41 (31.15-31.64)
16		100% (20/20)			33.95 (33.40-34.62)
8		100% (20/20)			34.93 (34.09-36.46)
4		95% (19/20)			36.24 (34.59-37.77)
2		15% (3/20)			37.92 (36.61-40)
1		0% (0/20)			38.71 (36.95-40)
TGEV S-gene IVT RNA					
8 x 10^7			100% (3/3)		9.82 (9.59-10.02)
8 x 10^6			100% (3/3)		14.18 (13.96-14.50)
8 x 10^5			100% (3/3)		17.52 (17.29-17.69)
8 x 10^4			100% (3/3)		21.63 (21.53-21.74)

8 x 10^3	100% (3/3)		25.28 (25.21-25.34)
8 x 10^2	100% (3/3)		28.81 (28.75-28.85)
8 x 10^1	100% (3/3)		32.71 (32.67-32.77)
16	95% (19/20)		35.75 (34.83-37.34)
8	25% (5/20)		37.47 (35.87-38.75)
4	0% (0/20)		39.16 (37.14-40)
2	0% (0/20)		39.79 (37.95-40)
1	0% (0/20)		39.96 (39.61-40)
SADS-CoV N-gene IVT RNA			
1.36 x 10^8		100% (3/3)	11.11 (10.89-11.27)
1.36 x 10^7		100% (3/3)	13.56 (13.47-13.62)
1.36 x 10^6		100% (3/3)	17.26 (17.16-17.34)
1.36 x 10^5		100% (3/3)	20.47 (20.42-20.51)
1.36 x 10^4		100% (3/3)	23.87 (23.84-23.90)
1.36 x 10^3		100% (3/3)	27.27 (27.15-27.35)
1.36 x 10^2		100% (3/3)	30.65 (30.54-30.75)
27.2		100% (20/20)	32.91 (32.35-34.58)
13.6		100% (20/20)	34.23 (33.54-35.21)
6.8		100% (20/20)	35.41 (34.53-36.76)
3.4		85% (17/20)	36.38 (35.25-38.55)
1.7		35% (7/20)	37.93 (35.94-40)

* The PEDV/PDCoV/TGEV/SADS-CoV/XIPC 5-plex PCR Ct <37 was considered positive for each virus

Table 9. Specimen types of 219 clinical samples and Ct ranges of positive samples tested by the reference PEDV/PDCoV/TGEV PCR

Specimen type	Number	Reference PCR - PEDV Positive (Ct <36)		Reference PCR - PDCoV Positive (Ct <36)		Reference PCR - TGEV Positive (Ct <36)	
		Number	Ct Range	Number	Ct range	Number	Ct range
Fecal swab	54	21	16.6-33.2	13	15.5-35.7	3	25.44-31.1
Feces	53	20	14.57-30.2	32	14.18-36	9	14.9-28.4
Oral fluid	82	33	17.8-36	36	17.8-36	0	
Small intestine	30	22	15.2-35.2	1	20.7	0	
Total	**219**	**96**		**82**		**12**	

Table 10. Diagnostic performance of PEDV/PDCoV/TGEV/SADS-CoV/XIPC 5-plex PCR in comparison with the reference PEDV/TGEV/PDCoV PCR on clinical samples*

		Reference PCR - **PEDV**		
		Positive	Negative	Total
5-plex PCR - **PEDV**	Positive	95	6	101
	Negative	1	117	118
	Total	96	123	219

Sensitivity 98.96%; specificity 95.12%; agreement 96.80%

		Reference PCR - **PDCoV**		
		Positive	Negative	Total
5-plex PCR - **PDCoV**	Positive	82	3	85
	Negative	0	134	134
	Total	82	137	219

Sensitivity 100%; specificity 97.81%; agreement 98.63%

		Reference PCR - **TGEV**		
		Positive	Negative	Total
5-plex PCR - **TGEV**	Positive	12	0	12
	Negative	0	207	207
	Total	12	207	219

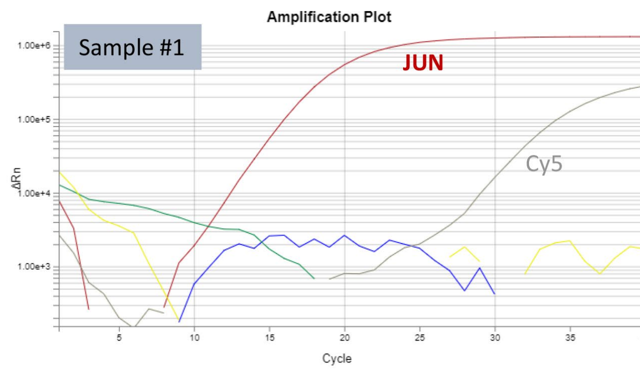
Sensitivity 100%; specificity 100%; agreement 100%

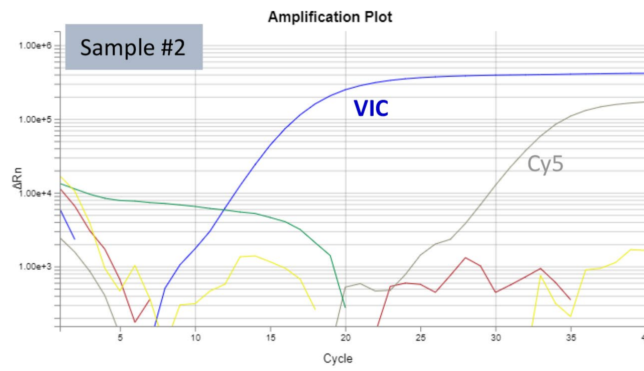
* Ct <37 was considered positive for the PEDV/PDCoV/TGEV/SADS-CoV/XIPC 5-plex and Ct <36 was considered positive for the reference PEDV/TGEV/PDCoV PCR

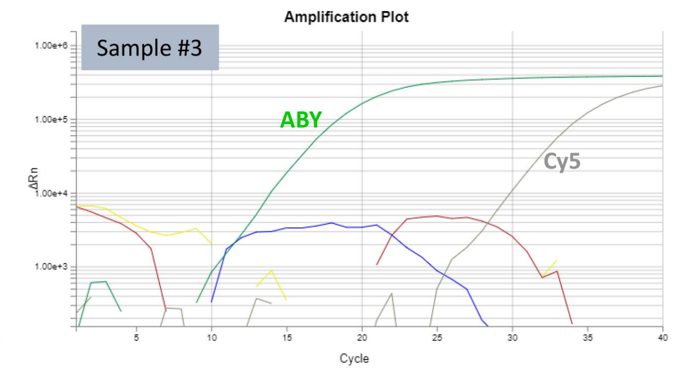
Table 11. Discrepancies on clinical samples between the PEDV/PDCoV/TGEV/SADS-CoV/XIPC 5-plex PCR and the reference PEDV/TGEV/PDCoV PCR*

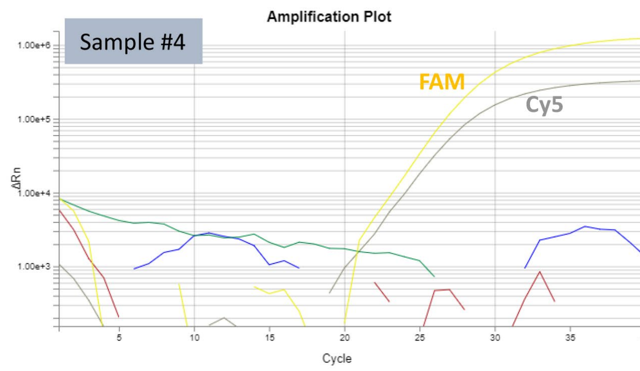
Sample ID	Specimen	Reference PCR			5-plex PCR			
		PEDV	PDCoV	TGEV	PEDV	PDCoV	TGEV	SADS-CoV
Sample_#23	Fecal swab	36.4	≥40	≥40	36.6	≥40	≥40	≥40
Sample_#24	Fecal swab	36.4	≥40	≥40	35.1	≥40	≥40	≥40
Sample_#27	Fecal swab	39.4	≥40	≥40	36.4	≥40	≥40	≥40
Sample_#75	Feces	36.3	34.6	≥40	35.4	31.4	≥40	≥40
Sample_#138	Oral fluid	35.0	≥40	≥40	≥40	≥40	≥40	≥40
Sample_#144	Oral fluid	38.5	≥40	≥40	35.6	≥40	≥40	≥40
Sample_#154	Oral fluid	≥40	28.6	≥40	35.8	27.4	≥40	≥40
Sample_#113	Oral fluid	21.5	37.0	≥40	20.2	33.5	≥40	≥40
Sample_#115	Oral fluid	24.6	36.8	≥40	23.8	36.4	≥40	≥40
Sample_#145	Oral fluid	39.0	37.7	≥40	39.2	36.0	≥40	≥40

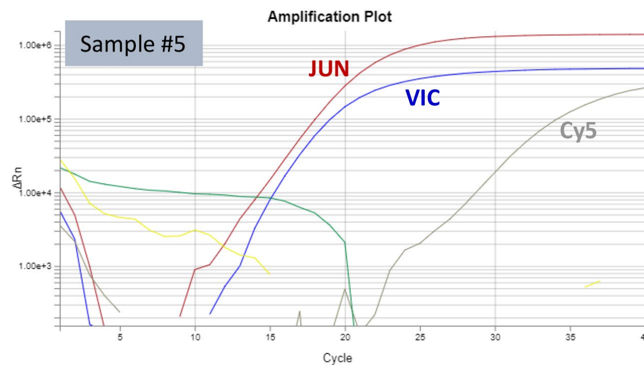
* Ct <37 was considered positive for the PEDV/PDCoV/TGEV/SADS-CoV/XIPC 5-plex and Ct <36 was considered positive for the reference PEDV/TGEV/PDCoV PCR

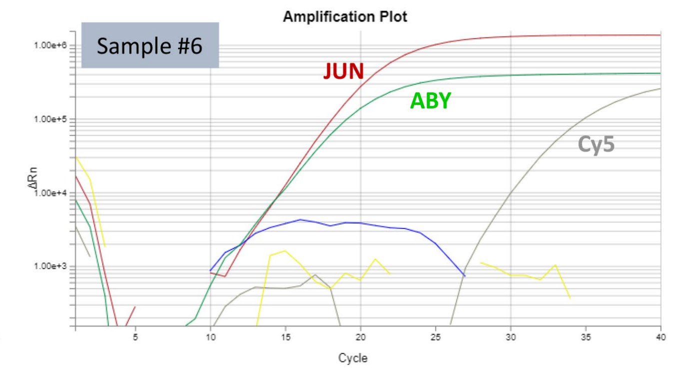

Amplification Plot
Sample #1
JUN
Cy5
ΔRn
Cycle

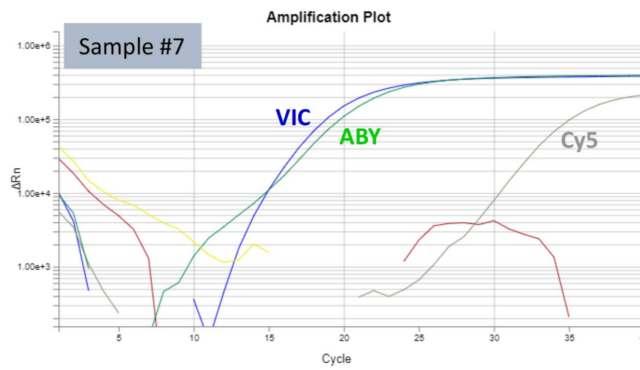

Amplification Plot
Sample #2
VIC
Cy5
ΔRn
Cycle

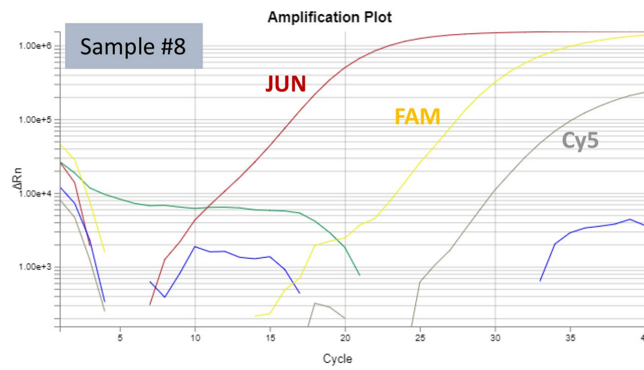

Amplification Plot
Sample #3
ABY
Cy5
ΔRn
Cycle

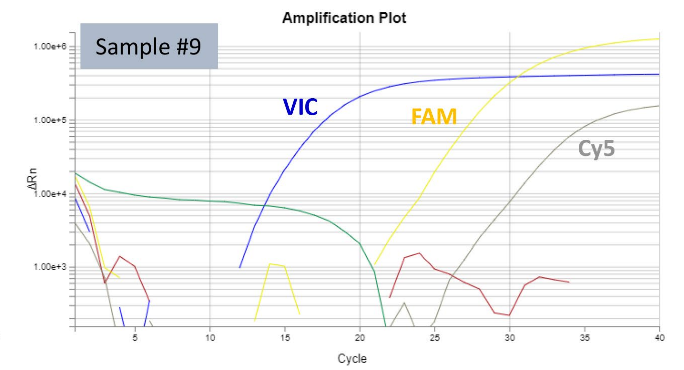

Amplification Plot
Sample #4
FAM
Cy5
ΔRn
Cycle


Amplification Plot
Sample #5
JUN
VIC
Cy5
ΔRn
Cycle


Amplification Plot
Sample #6
JUN
ABY
Cy5
ΔRn
Cycle


Amplification Plot
Sample #7
VIC
ABY
Cy5
ΔRn
Cycle


Amplification Plot
Sample #8
JUN
FAM
Cy5
ΔRn
Cycle


Amplification Plot
Sample #9
VIC
FAM
Cy5
ΔRn
Cycle

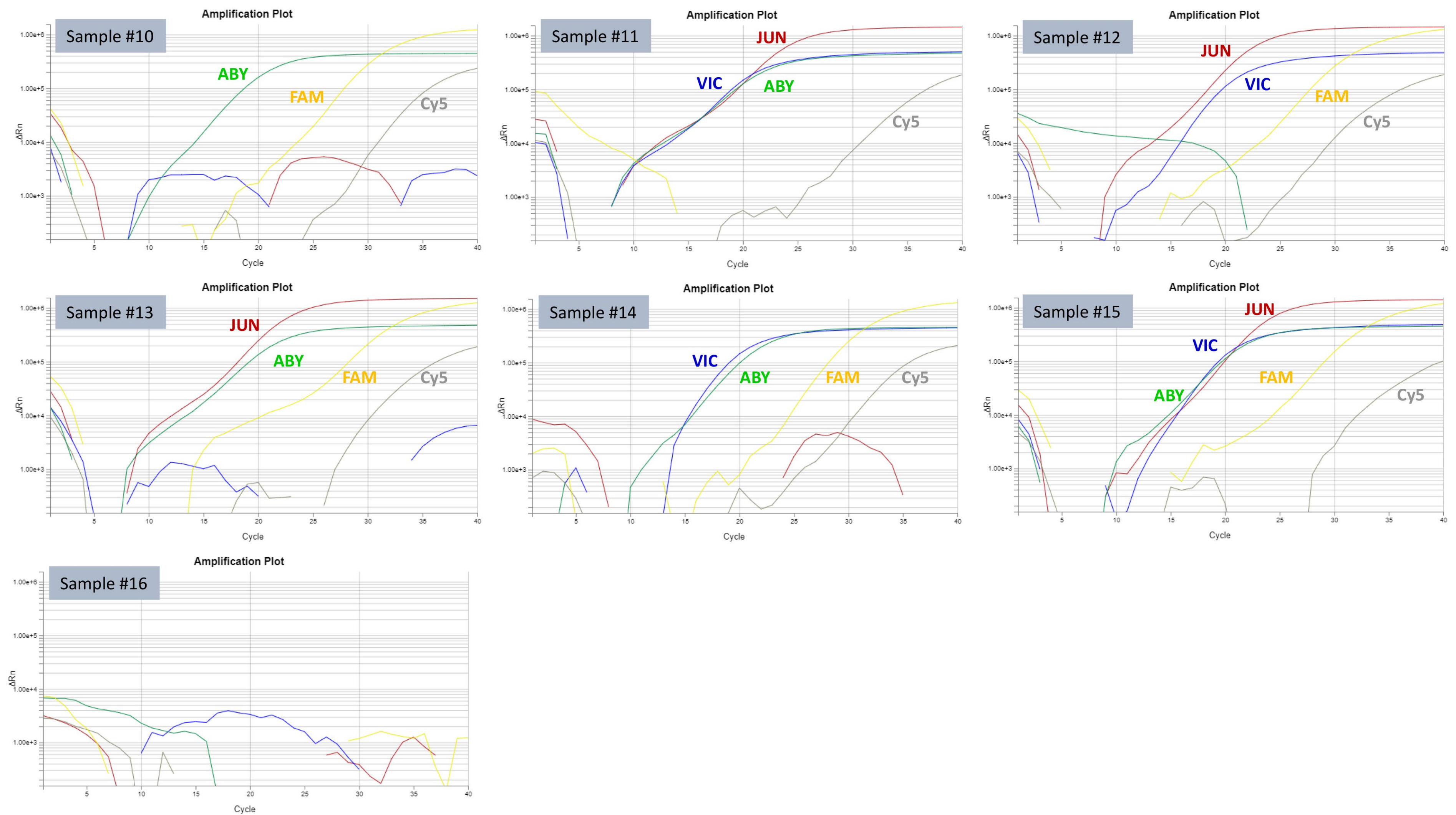


Figure 1. Amplification plots of 16 samples tested by PEDV/PDCoV/TGEV/SADS-CoV/XIPC 5-plex PCR. The amplification curves corresponding to each fluorescence dye (JUN, VIC, ABY, FAM, and Cy5) are labeled accordingly. Sample #1: PEDV. Sample #2: TGEV. Sample #3: PDCoV. Sample #4: SADS-CoV. Sample #5: manual mixture of PEDV and TGEV at volume ratio of 1:1. Sample #6: manual mixture of PEDV and PDCoV at volume ratio of 1:1. Sample #7: manual mixture of TGEV and PDCoV at volume ratio of 1:1. Sample #8: manual mixture of PEDV and SADS-CoV at volume ratio of 1:1. Sample #9: manual mixture of TGEV and SADS-CoV at volume ratio of 1:1. Sample #10: manual mixture of PDCoV and SADS-CoV at volume ratio of 1:1. Sample #11: manual mixture of PEDV, TGEV and PDCoV at volume ratio of 1:1:1. Sample #12: manual mixture of PEDV, TGEV and SADS-CoV at volume ratio of 1:1:1. Sample #13: manual mixture of PEDV, PDCoV and SADS-CoV at volume ratio of 1:1:1. Sample #14: manual mixture of TGEV, PDCoV and SADS-CoV at volume ratio of 1:1:1. Sample #15: manual mixture of PEDV, TGEV, PDCoV, and SADS-CoV at volume ratio of 1:1:1:1.