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Development of a triplex real-time RT-PCR assay for detection and differentiation of three US genotypes of porcine hemagglutinating encephalomyelitis virus

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ABSTRACT

Porcine hemagglutinating encephalomyelitis virus (PHEV) is a single-stranded, positive-sense RNA virus. PHEV mainly causes two types of clinical manifestations representing vomiting and wasting and encephalomyelitis in piglets. However, our recent findings provide strong evidence that PHEV can also cause respiratory disease in older pigs. Genomic analysis of new PHEV strains identified in our former study further classifies PHEV into three genotypes. Detection and differentiation of these new mutants are critical in monitoring PHEV evolution in the field. In the present study, we report the development of a triplex real-time RT-PCR assay for detection and differentiation of three PHEV genotypes, 1, 2, and 3. Three sets of primers and probes were designed; one set of primers and probe targeting the conserved regions of the 3′ end nucleocapsid for detection of all three genotypes and another two sets of primers and probes targeting the regions of NS2 with different patterns of deletions for detection of both genotypes 1 and 3, or genotype 3 only. Genotype 1 was positive when two probe dyes showed signals, genotype 2 was positive when only one probe dye showed a signal, and genotype 3 was positive when all three probes showed signals. The detection limit of the developed triplex real-time RT-PCR was as low as 8 or 9 DNA copies for three sets of primers and probes. The specificity test showed no cross reaction with other porcine viruses. Positive field-samples were correctly typed by this new assay, which was further confirmed by DNA sequencing. The triplex real-time RT-PCR provides a rapid and sensitive method to detect and differentiate all three US genotypes of PHEV from clinical samples.

1. Introduction

Porcine hemagglutinating encephalomyelitis virus (PHEV) is one of six known porcine coronaviruses (CoVs) causing diseases in pigs (Gong et al., 2017; Wang and Zhang, 2016). PHEV typically affects pigs less than three weeks of age and the clinical syndromes include the vomiting and wasting disease (VWD) and encephalomyelitis (Quiroga et al., 2008). PHEV was first isolated from suckling piglets suffering from encephalomyelitis in Canada in 1962 (Greig et al., 1962). PHEV has since been identified in many countries of Europe, Asia, and America (Cartwright et al., 1969; Dong et al., 2014; Forman et al., 1979; Hirahara et al., 1987; Mengeling, 1975; Pensaert and Callebaut, 1974; Quiroga et al., 2008; Rho et al., 2011). Although PHEV infection has been endemic in different countries for decades and the primary route of PHEV infection is through upper and lower respiratory tracts, only few reports suggest an association of the virus with respiratory disease in swine (Cutlip and Mengeling, 1972). Recently, we have demonstrated that PHEV caused an influenza-like illness (ILI) in affected pigs and provided evidence for the role of PHEV as a respiratory pathogen (Lorbach et al., 2017). In that study, a significantly higher positive rate 38.7% (108 of 279 pigs) was observed in pigs at the Michigan fairs compared to 4.1% (23 of 560 pigs) in Indiana and Ohio, indicating the PHEV-associated epizootic behavior in the Michigan fairs (Lorbach et al., 2017).

PHEV is a single-stranded positive-sense RNA virus and belongs to the *Betacoronavirus* genus of the *Coronaviridae* family. The first two

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thirds of the PHEV genome contain two large open reading frames (ORF1a and ORF1b) encoding the replicases, and the remaining part of the genome encodes six structural proteins (hemagglutinin-esterase protein, HE; spike glycoprotein, S; envelope protein, E; membrane protein, M; nucleocapsid protein, N, and N2) and three non-structural (NS) proteins (NS2, NS4.9, and NS12.7). Previous genomic characterization and analysis reveal that PHEV is closely related to bovine CoV and human CoV OC43, and have identified truncated NS2 and NS4.9 genes in the PHEV-VW572 strain (Vijgen et al., 2006). Prior to our study (Lorbach et al., 2017), only one complete PHEV genome sequence (PHEV-VW572) was available in GenBank. We completed the full genomic sequencing of 10 PHEV isolates, and the analyses of complete genomic sequences showed that PHEV formed three distinct genotypes. Interestingly, genotypes 1 had deletions in NS2 and NS4.9, while genotype 3 showed no deletion and genotype 2 has a large deletion in the NS2 gene (Lorbach et al., 2017). The prevalence and distribution of three genotypes remain unknown in pigs in the United States.

The traditional real-time RT-PCR (rRT-PCR) test currently used in diagnostic laboratories does not differentiate emerged PHEV from classical PHEV strains (Lorbach et al., 2017). The triplex rRT-PCR developed in the present study will fulfill this purpose and can be used to monitor PHEV of different genetic genotypes and differentiate between them.

2. Methods

2.1. Primer and probe design

Complete genomic sequences of all PHEV strains available in the GenBank database were collected and aligned using ClustalW of Mega 7.0.26 program. Based on the conserved and variable regions of sequences, three sets of primers and probes were designed; the first set targeting the 3′ non-coding region and N to detect all three genotypes (N-G123), the second set targeting the NS2 region to detect genotypes 1 and 3 (NS2-G13) that have deletions for genotype 2, and the third set targeting the regions of NS2 for genotype 3 (NS2-G3) that there are deletions for genotypes 1 and 2 (Fig. 1). The probes were labeled with different dyes for multiplexing purpose (Table 1).

2.2. RNA extraction and quantification

RNA was extracted using QIAGEN One-For-All Vet Kit (Valencia, CA, USA) according to the manufacturer's instructions. The extracted RNA was eluted in 75 µl of elution buffer and stored at -80°C until use. Concentrations of RNA samples were measured using Qubit 3 Fluorometer.

2.3. rRT-PCR assay

Qiagen One Step RT-PCR kit (Valencia, CA, USA) was used for amplification in the ABI7500 Real-Time PCR System (Applied Biosystems, CA, USA). The assay was performed in a 25-µl reaction mixture containing 5 µl of 5 × Reaction buffer, 1 µl of each primer (10 µm), 1 µl of each fluorogenic probe (5 µm), 1 µl of dNTP (10mMol), 1 µl of enzyme Mix, 2.5 µl of purified RNA, and an appropriate volume of RNase-free distilled water (dH2O). The thermocycler amplification conditions were 50 °C for 30 min, 94 °C for 15 min, and 45 cycles of 95 °C for 15 s, 58 °C for 60 s, and 72 °C for 10 s.

2.4. Sensitivity of the triplex rRT-PCR assay

RNA was extracted from positive samples with known genotypes of 15SW1362 (genotype 1), 15SW1582 (genotype 2), and 15SW25049 (genotype 3), and then quantified using Qubit 3 fluorometer. The N gene was amplified using RNA of 15SW1582 (genotype 2) and the primer set of PHEV N F PCR and PHEV NCR R, and the NS2 gene were amplified using RNA of above three samples and the primer set of PHEV Poly-F PCR and PHEV HE-R PCR (Table 1). The PCR products were cloned into the pCR 2.1 vector (Invitrogen, Carlsbad, CA, USA). The detection limit of the triplex rRT-PCR assay was determined by testing 10-fold serially diluted positive PHEV RNAs (15SW1362 and 15SW25049) and plasmid DNAs (pCR 2.1-15SW1582-N, pCR 2.1-15SW1362-NS2, and pCR 2.1-15SW25049-NS2) in duplicate.

2.5. Specificity of the triplex rRT-PCR assay

Intra-specificity of the triplex real time RT-PCR assay was determined using a single probe of N-G123, NS2-G13, and NS2-G3 for detection of all three genotypes, both genotype 1 and 3, and genotype 3 only, respectively. Inter-specificity of triplex real time RT-PCR assay was examined using various swine viruses available in our laboratory. These viruses include porcine reproductive and respiratory syndrome virus, swine influenza virus (H3N2), transmissible gastroenteritis virus, porcine epidemic diarrhea virus, porcine deltacoronavirus, porcine circovirus 2, and senecavirus A. In the assay, 2.0 µl of RNA or DNA samples were used, 2.0 µl of genotype 3 strain 15SW25049 was used as a positive control, and 2.0 µl distilled water was used as a negative control.

2.6. Application of the triplex real-time RT-PCR

Positive controls for each genotype were used in each run of the triplex real-time RT-PCR. Nasal swab and nasal wipe samples were collected from exhibition swine at shows during 2015 – 2017. Seventy-five samples testing positive for PHEV were selected for this study and processed for RNA extraction. RNA samples were first tested for PHEV by a singleplex rRT-PCR as previously reported (Lorbach et al., 2017). If a test appeared positive, the triplex rRT-PCR was used to differentiate three genotypes of PHEV and a conventional PCR using a primer set of PEHV Poly-F PCR PEHV HE-R PCR (Table 1) was applied to amplify NS2 region. The amplicons were subjected to DNA sequencing using a Sanger method (ACGT, Inc.) for confirmation.

3. Results

3.1. Primer-probe set selection

Based on the sequence alignment and analyses of all complete genome sequences of different genotypes, a conserved region in the 3′ end of N gene was selected for detection of all three genotypes of PHEV, whereas NS2 gene with different patterns of deletions was used to design two sets of primers and probes to detect both genotypes 1 and 3, or genotype 3 only (Fig. 1). If only one FAM signal is detected, it indicates genotype 2; if two signals (FAM and CY5) are detected, it indicates genotype 1; and if all three signals (FAM, CY5, JOE) are detected, it indicates genotype 3 of PHEV (Table 1).

3.2. Specificity of the triplex rRT-PCR assay

The triplex RT-PCR assay specifically detected genotype 2 by the FAM probe only, genotype 1 by the FAM and CY5 probes, and genotype 3 by the FAM, CY5, and JOE probes. By contrast, the triplex RT-PCR did not cross-react with any other swine viruses (porcine reproductive and respiratory syndrome virus, swine influenza virus (H3N2), transmissible gastroenteritis virus, porcine epidemic diarrhea virus, porcine deltacoronavirus, porcine circovirus 2, and senecavirus A) used in the study.

3.3. Detection limit

The sensitivity of the triplex rRT-PCR was determined through 10-fold serial dilutions of RNAs of known PHEV genotypes (15SW1362 for

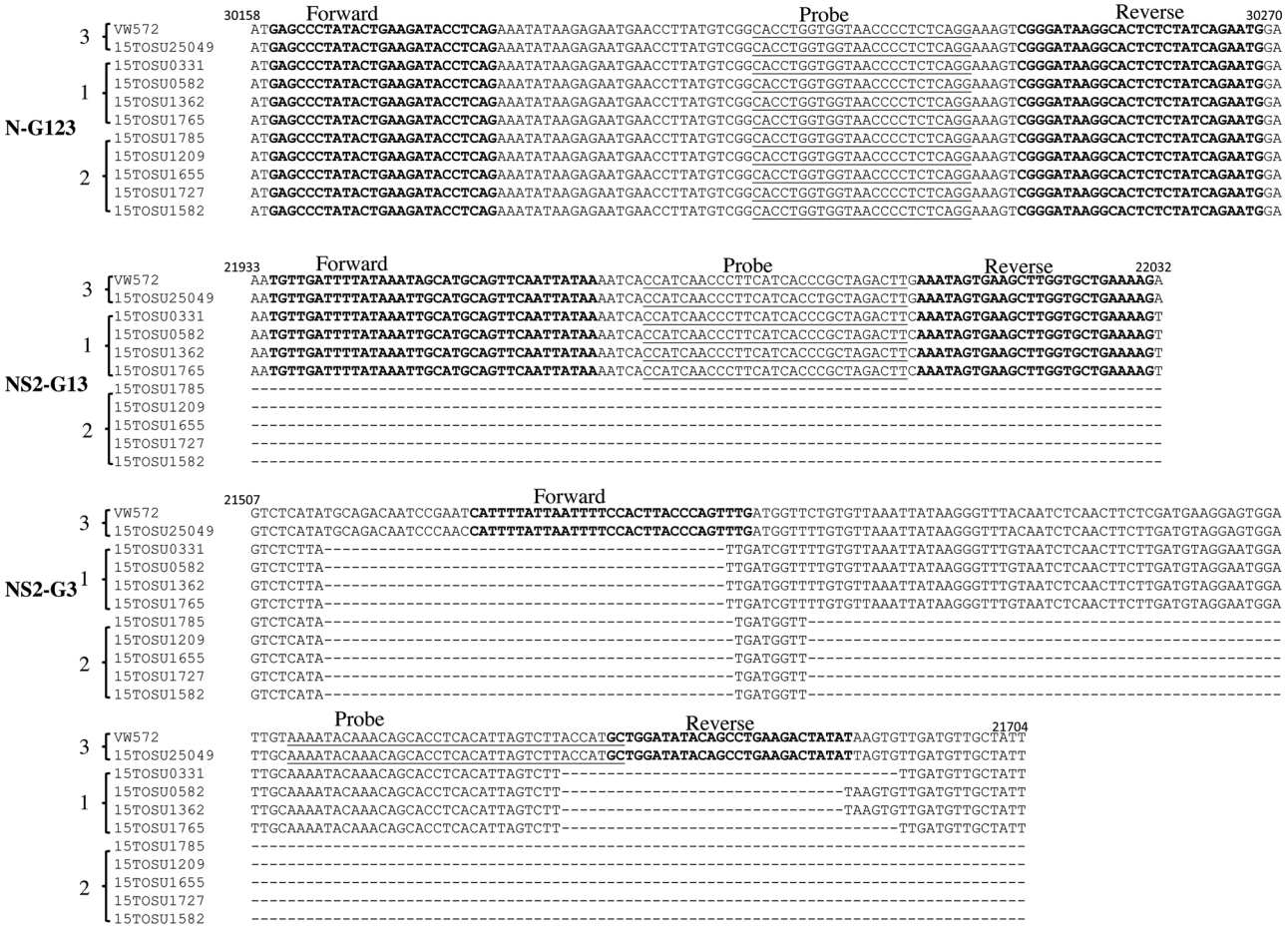

Fig. 1. Consensus sequence alignment of PHEVs of genotype 3 strains VW572 (accession no. DQ011855) and 15TOSU25049 (accession no. KY419103); genotype 1 strains 15TOSU0331 (accession no. KY419104), 15TOSU0582 (accession no. KY419105), 15TOSU1362 (accession no. KY419110), and 15TOSU1765 (accession no. KY419112); genotype 2 strains 15TOSU1785 (accession no. KY419106), 15TOSU1209 (accession no. KY419107), 15TOSU1655 (accession no. KY419109), 15TOSU1727 (accession no. KY419111), and 15TOSU1582 (accession no. KY419113) in the amplified regions of 3′ end N gene and NS2, including the primer (bold) and probe (underline) target sequences. Nucleotide numbering of PHEV in 3′ end N region and NS2 is indicated based on the complete genome sequence of VW572.

G123 and G13, and 15SW25049 for G3) and plasmids pCR 2.1-15SW1582-N for G123, pCR 2.1-15SW1362-NS2 for G13, and pCR 2.1-15SW25049-NS2 for G3 in duplicate. The detection limit was 0.0001 ng/μl and 9 DNA genome copies for N-G123 with Ct value 37 as a cutoff, and 0.001 ng/μl and 8 DNA genome copies for NS2-G13 and –G3 with Ct values 35 and 38 as cutoffs, respectively (Fig. 2). Both replicates of N-G123, NS2-G13, and NS2-G3 produced positive results at 8 or 9 DNA copies. There are strong linear correlations ($r^2 > 0.97$) between C_t values and amount of viral RNA and between C_t values and corresponding amount of plasmid copy numbers for different targets (Fig. 2A, B, and C).

3.4. Genotyping clinical samples

A total of 75 positive samples were tested by the singleplex rRT-PCR and were run again by the triplex real-time RT-PCR. Fifteen samples appeared positive for genotype 1, 39 samples were positive for genotype 2, and 21 were positive for genotype 3. The Sanger sequencing using PEHV Poly-F PCR-PEHV HE-R PCR primer set confirmed the genotyping results. To be noted, 8 out of 15 genotype 1 strains have a

Table 1
Primers and probes used in the study.

Name	Primer/probe sequence	Amplicon size (bp)
PHEV N F-G123	GAGCCCTATACTGAAGATACCTCAG	
PHEV NCR R-G123	TGATAGAGAGTGCCTTATCCCG	109
PHEV N Probe-G123	FAM-CACCTGGTGGTAACCCCTCTCAGG-3' Iowa Black® FQ	
PHEV NS2-F-G13	TGTTGATTTTATAAATWGCATGCAGTTCAATTATAA	
PHEV NS2-R-G13	CTTTTCAGCACCAAGCTTCACTATTT	97
PHEV NS2-Probe-G13	CY5-CCATCAACCCTTCATCACCYGCTAGACTT-3' Iowa Black® RQ	
PHEV NS2-F-G3	CCATTTTATTAATTTTCCACTTACCCAGTTTG	
PHEV NS2-R-G3	ATATAGTCTTCAGGCTGTATATCCAGC	155
PHEV NS2-Probe-G3	JOE-AAAATACAAACAGCACCTCACATTAGTCTTACCATGC-3' Iowa Black® FQ	
PEHV Poly-F PCR	GAGCAGACCAGATTAATGATATGG	1077 (genotype 1) 393 (genotype 2)
PEHV HE-R PCR	GAATCACACAGAGCAGGATTAAGG	1171 (genotype 3)
PEHV N-F PCR	ACTTGGCAAGGATGCCACTAAG	692 (with PHEV NCR R)

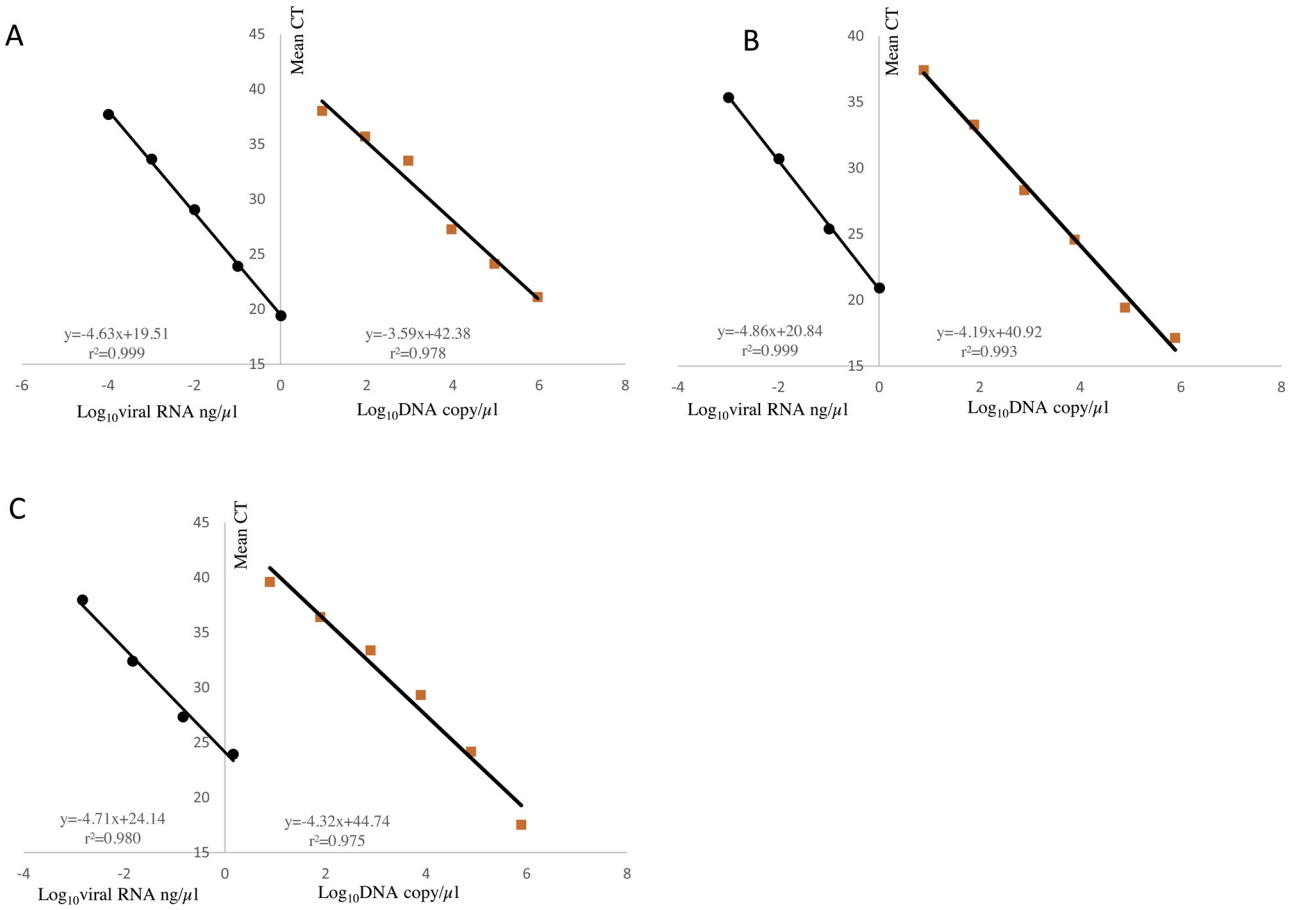

Fig. 2. Standard curves for the triplex real-time RT-PCR assay. (A) Viral 15SW1362 RNA (left) and plasmid pCR 2.1-15SW1582-N DNA (right) standard curves for PHEV genotypes 1, 2, and 3, y = −4.63x + 19.51, r2 = 0.999 and y = −3.59x + 42.38, r2 = 0.978, (B) Viral 15SW1362 RNA (left) and plasmid pCR 2.1-15SW1362-NS2 DNA (right) standard curves for PHEV genotypes 1, and 3, y = −4.86x + 20.84, r2 = 0.999 and y = −4.19x + 40.92, r2 = 0.993, and (C) Viral 15SW25049 RNA (left) and plasmid pCR 2.1-15SW25049-NS2 DNA (right) standard curves for PHEV genotype 3, y = −4.71x + 24.14, r2 = 0.980 and y = −4.32x + 44.74, r2 = 0.975.

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                21501
VW572           ATGGCCGTCT CATATGCAGA CAATCCGAAT CATTTTATTA ATTTTCCACT TACCCAGTTT GATGGTTCTG TGTTAAATTA TAAGGGTTTA CAATCTCAAC TTCTCGATGA AGGAGTGGAT
15TOSU25049     ATGGCCGTCT CATATGCAGA CAATCCCAAC CATTTTATTA ATTTTCCACT TACCCAGTTT GATGGTTTTG TGTTAAATTA TAAGGGTTTA CAATCTCAAC TTCTTGATGT AGGAGTGGAT
15TOSU1362      ATGGCCGTCT CTTAT----- ---------- ---------- ---------- ---------T GATGGTTTTG TGTTAAATTA TAAGGGTTTG TAATCTCAAC TTCTTGATGT AGGAATGGAT
15TOSU1765      ATGGCCGTCT CTTAT----- ---------- ---------- ---------- ---------T GATCGTTTTG TGTTAAATTA TAAGGGTTTG TAATCTCAAC TTCTTGATGT AGGAATGGAT
16SW4347        ATGGCCGTCT CATATGCAGA TAATCTGAAC CATTTTATTA ATTTTCCACT TACCCAGTTT GATGGTTTTG TGTTAAATTA ---------- ---------- ---------- ---------- ----------
15TOSU1785      ATGGCTGTCT CATAT----- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------

VW572           TGTAAAATAC AAACAGCACC TCACATTAGT CTTACCATGC TGGATATACA GCCTGAAGAC TATATAAGTG TTGATGTTGC TATTCAAGAA GTTATTGATG ATATGCATTG GGGTGAGGGT
15TOSU25049     TGCAAAATAC AAACAGCACC TCACATTAGT CTTACCATGC TGGATATACA GCCTGAAGAC TATATTAGTG TTGATGTTGC TATTCAAGAA GCTATTGCTG ATATGCATTG GGGTGAGGGT
15TOSU1362      TGCAAAATAC AAACAGCACC TCACATTAGT CTT------- ---------- ---------- ----TAAGTG TTGATGTTGC TATTCAAGAA GTTATTGCTG ATATGCATTG GGGTGAGGGT
15TOSU1765      TGCAAAATAC AAACAGCACC TCACATTAGT CTT------- ---------- ---------- ---------- TTGATGTTGC TATTCAAGAA GTTATTGCTG ATATGCATTG GGGTGAGGGT
16SW4347        ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ATATGCATTG GGGTGAGGGT
15TOSU1785      ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------

VW572           TTTCAGATTA AGTTTGATAA CCCCCACATC CTAGGAAGAT GCTTAGTTTT AGACGTTAAA GGTGTAGAAG AGTTGCATGA TGATTTAGTT AATTACATTC GTGATAAAGG TTGTGTTGCC
15TOSU25049     TTTCAGATCA AGTTTGATAA CCCCCACATC CTAGGTAGAT GTTTAGTTTT AGACGTTAAA GGTATACAAA AATTGCATGA TGATTTAGTT AATCACATTC GTGCTAAAGG CTGTGTTGTT
15TOSU1362      TTTCAGATCA AGTTTGACAA -CCCTACATC CTAGGAATAT GCTTAGTTTT AGACGTTAAA GGTGTAGAAG AGTTGCATGA TGATTTATTT AATTACATTC GTGCTATAGG TTGTGTTGTC
15TOSU1765      TTTCAGATCA AGTTTGACAA -CCCTACATC CTAGGAATAT GCTTAGTTTT AGACGTTAAA GGTGTAGAAG AGTTGCATGA TGATTTATTT AATTACATTC GTGCTATAGG TTGTGTTGTC
16SW4347        TTTCAGATCA AGTTTGATAA CCCCCACATC CTAGGTAGAT GCTTAGTTTT AGACGTTAAA GGTGTACAAG AATTGCATGA TGATTTAGTT AATCACATTC GTTCTAAAGG CTGTGTTGTT
15TOSU1785      ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------

VW572           GACCAATCCA GAAAATGGAT TGGCCATTGC ACCATAGCCC AATTCACGGA TGCTGCACTT TCCATTAAAG AAAATGTTGA TTTTATAAAT AGCATGCAGT TCAATTATAA AATCACCATC
15TOSU25049     GACCAATCCA GAAAATGGAT TGGCCACTGC ACCATAGCTC AATTCTTGGA TGCTGCACTT TCTATTAAAG AAAATGTTGA TTTTATAAAT TGCATGCAGT TCAATTATAA AATCACCATC
15TOSU1362      GACCAATTCA GAAAATGGAT TGGCCACTGC ACCATAGCTC AATTCATGGA TGCTGCACTT TCTATTAAAG AAAATGTTGA TTTTATAAAT TGCATGCAGT TCAATTATAA AATCACCATC
15TOSU1765      GACCAATTCA GAAAATGGAT TGGCCACTGC ACCACAGCTC AATTCATGGA TGCTGCACTT TCTATTAAAG AAAATGTTGA TTTTATAAAT TGCATGCAGT TCAATTATAA AATCACCATC
16SW4347        GACCAATCCA GAAAATGGAT TGGCCACTGC ACCATAGCTC AATTTTTGGA TGCTGCATTT TCTATTAAAG AAAATGTTGA TTTTATAAAT TGCATGCAGT TCAATTATAA AATCACCATC
15TOSU1785      ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------
                                                                                                                                    22085
VW572           AACCCTTCAT CACCCGCTAG ACTTGAAATA GTGAAGCTTG GTGCTGAAAA GAAGGATGGT TTTTATGAAA CCGTAGTTAG CCATTGGATG GGAATTCGTC ATTAG-----
15TOSU25049     AACCCTTCAT CACCTGCTAG ACTTGAAATA GTGAAGCTTG GTGCTGAAAA GAAGGATGGT TTTTATGAAA CCGTAGTTAG CCATTGGATG GGAATTCGTC ATGAATAA--
15TOSU1362      AACCCTTCAT CACCCGCTAG ACTTCAAATA GTGAAGCTTG GTGCTGAAAA GTAGGATGGT TTTTATGAAA CCGTAGTTAG CCATTGGATG GGAATTCGTA ATGAATATAA
15TOSU1765      AACCCTTCAT CACCCGCTAG ACTTCAAATA GTGAAGCTTG GTGCTGAAAA GTAGGATGGT TTTTATGAAA CCGTAGTTAG CCATTGGATG GGAATTCGTA ATGAATATAA
16SW4347        AACCCTTCAT CACCTGCTAG ACTTGAAATA GTGAAGCTTG GTGCTGAAAA GAAGGATGGT TTTTATGAAA CCGTAGTTAG CCATTGGATG GTAATTCGTC ATGAATATAA
15TOSU1785      ---------- ---------- ---------- ---------- ---------- ----GATGGT T--------- ---------- ---------- ---------- ----------
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Fig. 3. Sequence alignment of NS2 genes of different PHEV genotypes including new PHEV genotype 1 variant 16SW4347. The deletion region of 16SW4347 was marked with a red-color frame. The nucleotide position was based on VW572 (accession no. DQ011855) (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.).

different deletion pattern from that originally identified (Fig. 3).

4. Discussion

In recent years, with the help of molecular technology, more novel coronaviruses have been identified in pigs. So far, there are six known porcine coronaviruses including transmissible gastroenteritis coronavirus (TGEV), porcine epidemic diarrhea virus (PEDV), porcine deltacoronavirus (PDCoV), porcine respiratory coronavirus (PRCV), bat HKU2-like porcine enteric alphacoronavirus (PEAV), and PHEV. These porcine coronaviruses cause neurologic (PHEV), digestive (TGEV, PEDV, PDCoV, PEAV, PHEV), and respiratory diseases (PRCV, PHEV). VWD and encephalomyelitis caused by PHEV are common and have experimentally been reproduced in pigs (Mengeling and Cutlip, 1976). In contrast, only a few studies including ours report that PHEV causes respiratory problems in pigs. In our previous study, we reported that influenza-like illness in market-age pigs was strongly linked to PHEV (Lorbach et al., 2017). Field and experiment infection studies are needed to elucidate the pathogenic mechanism of respiratory diseases caused by PHEV.

In addition to the uncommon clinical presentation of PHEV, we show that there are three different genotypes cocirculating in the field in US. Different deletion patterns (small, large, none) in NS2 are observed in genotypes 1, 2, and 3, respectively. Genotype 1 has a small deletion in NS 4.9. Genotype 2 was predominant in pigs with respiratory disease from Michigan fairs (Lorbach et al., 2017). It remains unknown if different genotypes have similar virulence.

To further type samples positive for PHEV, we have developed a triplex rRT-PCR using one FAM dye for genotype 2, the FAM and CY5 dyes for genotype 1, and the FAM, CY5, and JOE dyes for genotype 3. Our data indicate that the triplex RT-PCR is highly specific with no cross-reaction with other known swine viruses and is sensitive with a detection limit ranging between $0.0001 - 0.001$ ng/μl of viral RNA and 8–9 DNA genome copies. Furthermore, the developed assay correctly subtyped 75 PHEV-positive samples, which was confirmed by Sanger sequencing. It should be noted that genotype 2 strains previously identified in our previous study have defective NS2 gene, and 35 out of 39 genotype 2 strains detected in the present study have a larger deletion in NS2, resulting in the complete deletion of NS2. Furthermore, 8 out of 15 genotype 1 strains identified in the study have a different deletion pattern from other 7 strains in genotype 1 previously identified (Fig. 3).

Similar to the finding that genotype 2 was predominant in pigs of the Michigan fair, genotype 2 was also predominant (52%) in 75 samples tested in the present study, whereas positive rates for genotype 1 and 3 were 20% and 28%, respectively. Future studies are needed to address whether the large deletion in NS2 benefits virus replication or not. In contrast to conventional singleplex real time RT-PCR, the triplex real time RT-PCR that we have developed in this present study can be used to monitor PHEV genotypes and potentially to identify new variants if test results cannot be explained as expected.

Few limitations of our study need to be noted. For the specificity test, not all porcine coronaviruses were tested due to unavailability of PRCV and PEAV. Since PRCV is a spike deletion mutant of TGEV and the triplex assay developed did not show the cross reaction with TGEV, it should cross react with PRCV. For PEAV, blast search of sequences of primers and probes used in the triplex PCR showed no any hit to genome of PEAV, indicating that a cross reaction should not occur. The observed low efficiency of the triplex real-time RT-PCRs in the study was due to unknown factors including amount of PCR reagents and design of primers and probes.

In summary, we have developed a triplex real time RT-PCR to detect and differentiate all three genotypes of PHEV. This triplex RT-PCR assay is highly specific and sensitive. The developed assay may be used to monitor different genotypes circulating in the field.

Acknowledgements

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