MINI-REVIEW

Rapid and efficient detection methods of pathogenic swine enteric coronaviruses

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Abstract

Porcine enteric coronaviruses (CoVs) cause highly contagious enteric diarrhea in suckling piglets. These COV infections are characterized by clinical signs of vomiting, watery diarrhea, dehydration, and high morbidity and mortality, resulting in significant economic losses and tremendous threats to the pig farming industry worldwide. Because the clinical manifestations of pigs infected by different CoVs are similar, it is difficult to differentiate between the specific pathogens. Effective high-throughput detection methods are powerful tools used in the prevention and control of diseases. The immune system of piglets is not well developed, so serological methods to detect antibodies against these viruses are not suitable for rapid and early detection. This paper reviews various PCR-based methods used for the rapid and efficient detection of these pathogenic CoVs in swine intestines.

Key points

1. *Swine enteric coronaviruses (CoVs) emerged and reemerged in past years.*
2. *Enteric CoVs infect pigs at all ages with high mortality rate in suckling pigs.*
3. *Rapid and efficient detection methods are needed and critical for diagnosis.*

Keywords RT-PCR, detection methods · Swine Diarrhea · Pathogenic enteric coronaviruses

Introduction

The coronaviruses (CoVs) critically threaten human and animal health because infection with them results in respiratory or enteric tract diseases (Woo et al. 2012). For instance, pneumonia occurs in humans infected with the new CoV that emerged in China in the middle of Dec. 2019; the virus has subsequently spread to many countries worldwide where it threatens the health of humans, resulting in tremendous economic losses. CoVs are enveloped, positive-sense, single-stranded RNA viruses that possess a genome ranging from 25.4 to 31.7 kb; they belong to the order Nidovirales, family Coronaviridae, and subfamily Coronavirinae (Woo et al. 2012). Based on antigenic relationships, the classification of coronaviruses was traditionally divided into 3 genera, but they were replaced by the following four genera: *Alphacoronavirus* (Alpha-CoV), *Betacoronavirus* (Beta-CoV), *Gammacoronavirus* (Gamma-CoV), and *Deltacoronavirus* (Delta-CoV), which are based on genetic and antigenic characteristics (Woo et al. 2010). Epidemiological surveys have indicated that bats and birds seem to be natural reservoirs for Alpha- and Beta-CoVs and Gamma- and Delta-CoVs, respectively (Bolles et al. 2011; Woo et al. 2012). CoVs in four genera have been verified in a variety of species, e.g., canines, felines, and birds (Chan et al. 2013).

Six CoVs have been identified in swine (Table 1): porcine epidemic diarrhea virus (PEDV), transmissible gastroenteritis virus (TGEV), swine acute diarrhea syndrome coronavirus (SADS-CoV), and porcine respiratory CoV (PRCoV) in the Alpha-CoV genus, porcine hemagglutinating encephalomyelitis virus (PHEV) in the Beta-CoV genus, and porcine

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Table 1 Relevant information of swine coronaviruses

Viruses	Genus	First discovery	Tissue tropism	Clinical signs
TGEV	α-CoV	1946	Small intestines	Diarrhea, dehydration, weight loss
PEDV	α-CoV	1977	Small intestines	Diarrhea, dehydration, weight loss, death
SADS-CoV	α-CoV	2017	Small intestines	Diarrhea, dehydration, weight loss, death
PRCoV	α-CoV	1984	Respiratory tract	Coughing, mild fever, polypnea
PHEV	β-CoV	1957	Respiratory tract, central nervous system	Vomiting, wasting disease and/or encephalomyelitis
PDCoV	δ-CoV	2012	Small intestines	Diarrhea, dehydration, weight loss, death

TGEV, transmissible gastroenteritis virus; *PEDV*, porcine epidemic diarrhea virus; *CoV*, coronavirus; *SADS-CoV*, swine acute diarrhea syndrome coronavirus; *PRCoV*, porcine respiratory CoV; *PHEV*, porcine hemagglutinating encephalomyelitis virus; *PDCoV*, porcine deltacoronavirus; *α*, alpha; *β*, beta; *δ*, delta

deltacoronavirus (PDCoV) in the Delta-CoV genus (Jung et al. 2016; Pan et al. 2017; Pensaert and de Bouck 1978; Woo et al. 2012; Zhang 2016; Zhou et al. 2018). Among the six swine CoVs, TGEV, PEDV, PDCoV, and SADS-CoV are enteric viruses that cause diarrhea in the pig population, resulting in significant economic losses and tremendous threats to the pig industry worldwide. The four swine enteric CoVs causing highly contagious enteric diarrhea in neonatal and suckling piglets are clinically characterized by vomiting, watery diarrhea, dehydration, and high morbidity and mortality (Gong et al. 2017; Hsu et al. 2018). Because the clinical signs of pigs infected by these CoVs are very similar (Table 1), it is difficult to differentiate the specific pathogens based on clinical symptoms. Effective high-throughput detection methods are needed for their differential determination and would represent powerful tools to prevent and control diseases.

As far as we know, many standard detection methods can be used to distinguish between causative agents, including virus isolation, electron microscopy, virus neutralization, and indirect immunofluorescence assays. However, these methods are time-consuming, laborious, and not suitable for the early and rapid detection of the four swine enteric CoVs (Carman et al. 2002; Dulac et al. 1977; van Nieuwstadt et al. 1988). The enzyme-linked immunosorbent assay is a powerful and high-throughput method for detecting specific antibodies, but the immune system of piglets is not well developed, so serological methods for detecting antibodies against these viruses are also not suitable for rapid and early detection. Polymerase chain reaction (PCR) methods have been widely used to detect pathogens since the PCR was invented; PCR has proven to be powerful and convenient tools for precise detection of diarrheal pathogens in pig populations (Ben Salem et al. 2010; Collins et al. 2008; Kim et al. 2007). This paper reviews various PCR-based methods for the rapid and efficient detection of these pathogenic CoVs in swine intestines.

Pan-CoV RT-PCR assay for the detection of CoVs

Figure 1 summarizes the basic workflow for the detection of the swine enteric coronaviruses from clinical samples. Generally, porcine fecal or intestinal samples need to be suspended and homogenized in sterile PBS and centrifuged to remove debris. The supernatant should be collected and filtered through a 0.45-μm filter to remove the debris and some potential bacteria. The yield supernatant can be used to extract the total RNAs by Trizol reagent or RNA extraction kit. The total RNAs are used to reverse transcription by random primers to generate cDNA. This cDNA is employed as a template to do PCR amplification either by specific individual coronavirus or by pan-CoV primers first then followed by specific primers targeting individual swine enteric coronavirus when necessary. The PCR products are eventually subjected to DNA electrophoresis and analyzed under UV light to identify the desired bands.

The pan-CoV PCR method is a powerful tool for detecting all known and unknown CoVs; it is based on the conserved gene sequences among them (Moes et al. 2005). This assay is widely used to detect CoVs. Pan-CoV RT-PCR was employed to detect all known CoVs in the human respiratory tract (Vijgen et al. 2008) and to detect distinct Alpha-CoVs in five different bat species (Escutenaire et al. 2007; Lazov et al. 2018; Vijgen et al. 2008). For swine enteric CoVs, pan-CoV PCR also played an important role in identification. During the early stage of investigating cases of diarrhea in piglets in the USA caused by the PEDV variant and PDCoV, pan-CoV RT-PCR was applied to identify the causative agent together with electron microscopy and sequencing (Li et al. 2014; Stevenson et al. 2013). In addition, during the identification of piglet diarrhea disease caused by SADS-CoV in China, pan-CoV RT-PCR was employed (Pan et al. 2017). In 2018, Hu et al. reported an improved one-step pan-CoV RT-PCR
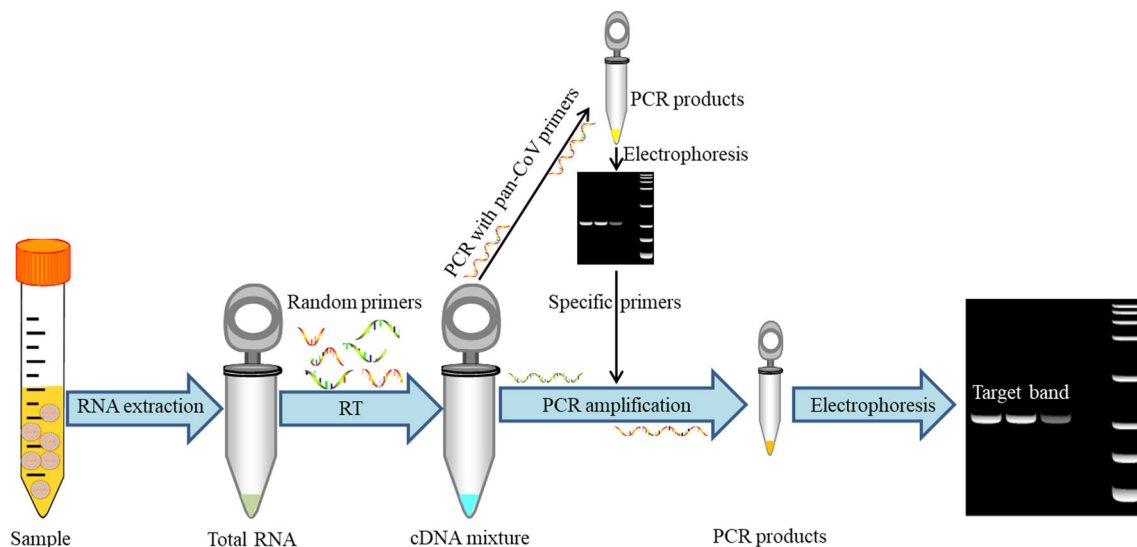
Fig. 1 The schematic diagram of the workflow for swine enteric coronavirus detection by RT-PCR. Porcine fecal or tissue samples are homogenized in sterile PBS and centrifuged to remove debris. The supernatant is filtered through a 0.45-μm filter and used to total RNA extraction. The total RNAs are subjected to reverse transcription using random primer to generate cDNA. With this cDNA as template, the PCR amplification is carried out either by specific individual coronavirus or by pan-CoV primers first then followed by specific primers targeting individual swine enteric coronavirus when necessary. The PCR products are subjected to DNA electrophoresis and observed under UV light to identify the desired bands

assay for the detection of known human and animal CoVs on the basis of the RNA-dependent RNA polymerase (RdRP) gene (Hu et al. 2018). Though pan-CoV PCR could detect all known CoVs in humans and animals, it could not make a differential detection, so it is not suitable for routine swine enteric CoV detection.

PEDV detection by PCR-based methods

Porcine epidemic diarrhea (PED) is an enteric disease caused by PEDV that can infect pigs of all ages with different levels of clinical signs of vomiting, diarrhea, dehydration, and weight loss, but the disease is much more severe in suckling piglets (Have et al. 1992; Shibata et al. 2000; Song and Park 2012; Sueyoshi et al. 1995). PED was first reported in England in 1971, but the PEDV was isolated for the first time in Belgium in 1978 (Pensaert and de Bouck 1978; Song and Park 2012); however, the epidemic was not controlled in Europe before 2000. In China, PEDV was first identified in the 1980s, after which it was reported in some Asian countries, e.g., Japan and Korea (Kusanagi et al. 1992; Song and Park 2012; Takahashi et al. 1983). In October 2010, a severe PED outbreak caused by a highly virulent PEDV variant emerged in southern China with high mortality ranging from 70 to 100%; the result was devastating damage to the pig farm industry and tremendous economic losses, and later, the PEDV variant spreads to other countries, e.g., USA, Canada, and Mexico (Chen et al. 2012; Chen et al. 2010; Ge et al. 2013; Jung and Saif 2015; Li et al. 2012; Ojkic et al. 2015;

Sun et al. 2012; Tian et al. 2014; Yang et al. 2014; Yang et al. 2013). Currently, PED remains a critical threat to the global swine industry.

For the early and rapid detection of PEDV, different types of PCR methods have been developed. A real-time reverse transcription recombinase polymerase amplification assay (RT-RPA) based on the nucleocapsid gene of PEDV was reported in 2018; the assay was able to detect 23 copies per reaction and was performed for 20 min at 40 °C (Wang et al. 2018). Based on the advantage of increased thermal conductivity of solid gold nanometal particles that could reduce nonspecific amplification and increase specific amplification, Yuan et al. developed a nanoparticle-assisted PCR assay for the detection of PEDV on the basis of the N gene in 2015, and the assay could detect 2.7×10^{-6} ng/μl of PEDV RNA (Yuan et al. 2015). Ren and Li designed six primers according to the sequence of the N gene and established a reverse transcription loop-mediated isothermal amplification (RT-LAMP) for the rapid detection of PEDV (within 50 min) at 63 °C. The detection limit of the method was 1×10^{-4} μg PEDV RNA per reaction (Ren and Li 2011). Wang et al. designed five primers on the basis of the N gene sequence of PEDV and established a reverse transcription cross-priming amplification-nucleic acid test strip (CPA-NAST) for the detection of PEDV. The method had high specificity for the detection of PEDV and had the same sensitivity as traditional RT-PCR; the detection limit was a 10^{-6} dilution of plasmid containing the target gene (Wang et al. 2016). Xing et al. developed an RNA extraction and transcription-free, nanoparticle-based PCR (NBP-PCR) method to specifically detect PEDV, and the sensitivity of

the method was 400-fold higher than that of conventional RT-PCR. In 153 fecal samples, the positive detection rate of NBP-PCR specific was much higher than that of conventional RT-PCR (5.88%) and SYBR Green real-time RT-PCR (Xing et al. 2016). Zhou et al. developed a conventional RT-PCR method, an SYBR Green I real-time RT-PCR, and TaqMan real-time RT-PCR reagents to detect the highly conserved M gene of PEDV; the detection limit of the TaqMan real-time RT-PCR was 10 copies/µl of the target gene, and the sensitivity of the TaqMan real-time RT-PCR was 100-fold and 10,000-fold higher than that of SYBR Green I real-time RT-PCR and conventional RT-PCR, respectively (Zhou et al. 2017).

In addition, there were variant PEDV strains circulating in the field and the PCR methods for differentiating them had been established. Song et al. analyzed pathogenicity and immunogenicity of PEDV strain designated DR13 in piglets, which was a highly Vero cell-adapted virus and could be employed as a vaccine candidate, and applied a restriction fragment length polymorphism (RFLP) assay to differentiate DR13 from wild-type virus based on the difference of open reading frame (ORF) 3 sequence (Song et al. 2003). In 2008, Lee et al. reported an RT-PCR-based RFLP assay targeting the N gene of PEDV to distinguish field strains of PEDV in Korea from an attenuated-live vaccine J-vac, which was used in pig population to prevent PEDV (Lee et al. 2008). For differentiation between attenuated-type PEDVs including attenuated DR13, KPED-9, and P-5V that were used as live virus vaccine and wild-type PEDVs including CV777, Brl/87, LZC, parent DR13, and field samples, Park et al. established an RT-PCR assay based on the difference of ORF 3 gene sequence of attenuated- and wild-type PEDV, in which 51 nucleotide deletions were found in all live PEDV vaccine (Park et al. 2008). In May 2013, the virulent strain of PEDV was verified in the USA resulting in significant economic losses in the swine industry. And a variant strain (OH851) of PEDV emerged in the USA in December 2013, which differs from the virulent strains of PEDV in the nucleotides of the 5′end of spike gene. To differentiate these two genotypes of PEDV circulating in the USA, Wang et al. reported a duplex probe-based real-time RT-PCR targeting the difference of spike gene among virulent and variant strains (Wang et al. 2014b). Sequence analysis of PEDV genome indicated that PEDV attenuated vaccine strains (e.g., the CV777 and ZJ08 in China, the P-5V in Japan, and KPED-9 and DR13 in South Korea in Asia) have 49 base pair deletion in the open reading frame 3 (ORF3); for differentiation of these cell-adapted vaccine strains from field strains, Zhu et al. developed a nanoparticle-assisted RT-PCR assay targeting the ORF3 (Zhu et al. 2016b). Because three major PEDV types have been identified in the USA after 2013 including the original US PEDV strains, the spike gene insertion-deletion PEDV strains, and the PC177 strain that possess a 197 amino acid-deletion in the S1region of spike

protein, Liu and Wang developed a reverse transcription-PCR method to differentiate these variants on the basis of differences in the S1 gene (Liu and Wang 2016). Since 2010, PEDV variants with base deletions and insertions in the S gene emerged in China and caused significant losses in piglets; Zhao et al. developed a TaqMan probe-based real-time PCR method for the detection of different PEDV variants and classical PEDV strains based on the sequence difference of the PEDV S gene, and the detection limit of the method was 5×10^2 target gene copies (Zhao et al. 2014). Su et al. established a duplex TaqMan probe real-time RT-qPCR method for detecting and differentiating classical and variant PEDVs targeting the difference in the S gene; the detection limit of the method was 4.8×10^2 genome copies/reaction for both the classical and variant PEDV. The results of clinical sample detection showed that the assay was more sensitive than conventional PCR, and variant PEDV was prevalent in China (Su et al. 2018). Due to the wide use of a live attenuated PEDV vaccine, classical and wild variant strains circulating in pig farms are common; therefore, He et al. established a multiplex RT-PCR to differentiate these strains based on the difference in S gene sequences, and the detection limit of the method was $1 \times 10^{1.7}$ TCID$_{50}$/100 µl for PEDV (He et al. 2019). Due to variant PEDV strains that emerged in China since 2010 and attenuated PEDV vaccines (e.g., CV777 strain) being widely used in China, Liu et al. reported a TaqMan probe-based real-time PCR to differentiate these virulent strains and attenuated vaccine strains based on the ORF3 deletion region to detect virulent PEDV strains in vaccinated pig population (Liu et al. 2019b).

TGEV detection by PCR-based methods

Transmissible gastroenteritis (TGE) caused by TGEV is an acute enteric diarrhea disease in pigs (Garwes 1988). TGEV was isolated for the first time in 1946, and then outbreaks of the virus occurred in many countries in America, Asia, and Europe (Doyle and Hutchings 1946; Kim et al. 2000; Stevenson et al. 2013). TGEV causes severe enteritis in piglets before weaning, and the clinical signs include diarrhea, vomiting, dehydration, and high mortality, resulting in significant economic losses (Ding et al. 2017; Penzes et al. 2001; Saif 1999).

For the early and rapid detection of TGEV, different types of PCR-based methods have been established. Chen et al. designed six specific primers targeting the nucleocapsid gene of TGEV and developed an RT-LAMP assay for the detection of TGEV, which involved incubation at 60 °C for 1 h. The sensitivity of RT-LAMP was comparable to that of nested PCR described by Rodríguez et al. in 2008, and it was 10 times more sensitive than the PCR reported by Paton et al. in 1997, which could detect 10 pg of RNA per reaction (Chen et al.

2010; Paton et al. 1997; Rodríguez et al. 2008). RT-LAMP for the detection of the TGEV targeting the N gene sequence by incubation at 60 °C for 30 min was reported by Li et al., and the sensitivity of RT-LAMP was much higher than that of a gel-based RT-PCR kit purchased from HaiGene Company, China (Li et al. 2012). Vemulapalli et al. described a TaqMan probe-based real-time RT-PCR that specifically amplified conserved S gene sequences. The detection limit of the method was 1 TCID$_{50}$/ml of TGEV RNA. The sensitivity of the assay was higher than that of the nested RT-PCR assay reported by Kim et al. (Kim et al. 2000; Vemulapalli et al. 2009). Wang et al. developed a rapid detection of TGEV using real-time reverse transcription recombinase polymerase amplification (RT-RPA) based on the spike gene, and the method could detect 100 copies of TGEV RNA after 20 min at 40 °C conditions (Wang et al. 2018). A real-time RT-PCR assay described by Vemulapalli et al. was compared with the RT-RPA method in the analysis of 76 clinical samples, and the two methods generated the same results (Vemulapalli et al. 2009; Wang et al. 2018). In the mid-1980s, PRCoV, which has a deletion of nucleotides of the S gene that is present in TGEV (Laude et al. 1993), was reported in Europe, North America, and Asia (Pensaert et al. 1986; Wesley et al. 1990). Paton et al. developed an RT-PCR assay to differentiate TGEV and PRCV based on the difference in the S gene of the two viruses (Paton et al. 1997).

PDCoV detection by PCR-based methods

PDCoV is a novel swine enteric diarrhea virus that causes severe diarrhea, vomiting, and dehydration in piglets (Janetanakit et al. 2016; Song et al. 2015). PDCoV was first discovered in samples from the healthy pig that were collected in 2009 in Hong Kong when a molecular surveillance study was performed (Woo et al. 2012). In February 2014, the virus was detected in piglets with severe diarrhea in the USA (Marthaler et al. 2014; Wang et al. 2014a). Subsequently, PDCoV was reported in South Korea, mainland China, and Thailand (Chen et al. 2015a; Janetanakit et al. 2016; Lee and Lee 2014; Song et al. 2015).

To detect the virus with precision, PCR-based methods have been created. In 2014, pigs on 5 farms in Ohio, USA, had clinical diarrheal disease. Wang et al. developed a one-step RT-PCR targeting membrane and nucleocapsid gene of delta-CoV and determined the causative agent as porcine delta-CoVs, but they did not evaluate the detection limit of the assay (Wang et al. 2014a). In 2015, Song et al. established a nested RT-PCR method on the basis of the nucleocapsid gene sequence of the PDCoV HKU15 strain to identify the causative agent causing acute diarrhea in a pig farm in Jiangxi, China, and the sensitivity of the assay was also not determined (Song et al. 2015). To analyze the characteristics of porcine delta-CoVs in the USA, Ma et al. established a real-time RT-PCR method for specific detection of the N gene, but the sensitivity of the assay was also not determined (Ma et al. 2015). For the analysis of pathogenicity and pathogenesis of a porcine delta-CoV cell culture isolate, Chen et al. developed an M gene-based real-time PCR method for detecting viral titers in different organs, but the sensitivity of the assay was not described in the publication (Chen et al. 2015b). Marthaler et al. developed a one-step probe-based real-time RT-PCR method targeting the M gene sequence of PDCoV, which was applied by the University of Minnesota Veterinary Diagnostic Laboratory. The detection limit of the assay was 2 viral RNA copies per reaction, but the specificity of the assay was not evaluated (Marthaler et al. 2014). The commercial reverse transcription-insulated isothermal PCR (RT-iiPCR) POCKIT™ methods (POCKIT™ PEDV Reagent Set and POCKIT™ PDCoV Reagent Set, GeneReach USA, Lexington, MA, USA) was used to analyze PEDV and PDCoV coinfection that occurred in diarrhea disease; Zhang et al. described two singleplex RT-iiPCR tests and a duplex real-time RT-PCR test for the detection of PEDV and PDCoV that were based on targeting the conserved M gene sequence. The detection limits of singleplex RT-iiPCR were 21 RNA copies per reaction for PEDV and 9 RNA copies per reaction for PDCoV, and those of the duplex RT-iiPCR were 7 RNA copies and 14 RNA copies per reaction for PEDV and PDCoV, respectively (Zhang et al. 2016).

SADS-CoV detection by PCR-based methods

SADS-CoV, also designated SeACoV or PEAV, is a novel porcine enteric diarrhea virus that can cause severe and acute diarrhea and rapid weight loss in piglets (Pan et al. 2017; Zhou et al. 2018; Zhou et al. 2017). SADS-CoV was identified for the first time in southern China in late 2017, and it caused more than 24,000 piglet deaths, resulting in significant economic losses (Gong et al. 2017). The clinical signs of infection with SADS-CoV are similar to those of other known swine enteric CoVs: TGEV, PEDV, and PDCoV (Dong et al. 2015; Sun et al. 2016).

For specific detection of SADS-CoV, Zhou et al. established an SYBR premix Ex TaqII-based real-time PCR on the basis of the RNA-dependent RNA polymerase (RdRp) gene for the detection of SADS-CoV; the sensitivity of which was not evaluated (Zhou et al. 2017). Zhou et al. developed a TaqMan-based real-time PCR assay for SADS-CoV detection based on the conserved sequence within the N gene; the detection limit of the assay was 3.0×10^1 copies/μl, and the sensitivity of the method was 10-fold higher than that of conventional PCR, which also targeted the N gene (Zhou et al. 2018).

Multiplex PCR-based methods for differential detection of pathogenic swine enteric CoVs

Because the clinical signs caused by the four enteric CoVs in piglets are similar to each other, it is very difficult or time-consuming to make a clear diagnosis of mixed infection in pigs using a single PCR method. Therefore, it is critical to developing a multiplex polymerase chain reaction (mPCR) in which two or more loci can be simultaneously detected in the same reaction (Chamberlain et al. 1988). TGEV and PEDV are traditional swine enteric diarrheal viruses, so several mPCR methods that can differentially detect the two viruses have been previously established. In 2001, Kim et al. reported a duplex RT-PCR assay to detect TGEV and PEDV in one PCR tube targeting the S gene of the two viruses, and the detection limit of the assay was 2 $TCID_{50}$/200 μl (Kim et al. 2001). In 2007, Kim et al. established a multiplex real-time RT-PCR method based on the nucleocapsid (N) gene for the simultaneous detection and quantification of TGEV and PEDV, and the detection limits of this method were 90 copies and 70 copies for TEGV and PEDV, respectively (Kim et al. 2007). Zhu et al. designed two pairs of primers on the basis of the N gene sequences of TGEV and PEDV and established a nanoparticle-associated PCR assay; the sensitivity of the assay was 10-fold higher than that of conventional PCR (Zhu et al. 2017).

Apart from the four enteric CoVs, several enteric diarrheal viruses have been discovered, including porcine sapovirus (PSaV), porcine norovirus (PNoV), porcine Teschen virus (PTV), porcine kobuvirus (PKV), Seneca Valley virus (SVV), porcine rotavirus (PRV), porcine reovirus (ReoV), porcine bocavirus (PBoV), and porcine astrovirus (PAstV). Therefore, some mPCR methods have been created to detect swine enteric diarrheal viruses, but they are not limited to enteric CoVs (Ding et al. 2019). Ben Salem et al. developed a nested RT-PCR method for the detection of PEDV, TGEV, and PRV based on the sequence of the TGEV Purdue strain (accession no. NC_002306), the PEDV strain CV777 (accession no. NC_003436), and the NSP5 gene of the PRV-A OSU strain (accession no. X15519), respectively. The detection limits of multiplex nested RT-PCR for TGEV and PEDV were 10^2 $TCID_{50}$/ml and 27.2 μg/μl of RNA, respectively (Ben Salem et al. 2010). In 2013, Zhao et al. developed a multiplex RT-PCR assay to identify PEDV, TGEV, PRV-A, and porcine circovirus 2 (PCV2) in one reaction, and the sensitivity of the assay for the detection of TGEV and PEDV was 10-fold lower than that of a single RT-PCR method (Zhao et al. 2013). Zhao et al. established a multiplex RT-PCR assay for rapid and differential diagnosis of PEDV, TGEV, PRV-A, and PCV2 targeting the S gene, segment 6 region, and ORF2 sequence, and the detection limits of the assay for TGEV and PEDV were 1.74×10^4 and 2.1×10^3 copies, respectively (Zhao et al. 2013). Liu et al. developed a multiplex PCR assay

to detect five diarrhea-related pig viruses: PEDV (nucleoprotein), TGEV (spike glycoprotein), PRV-A, porcine group C rotaviruses (PRV-C), and PCV2. The detection limits of the assay for PEDV and TGEV were 5 copies per reaction (Liu et al. 2019a). Wen et al. developed a multiplex real-time PCR method based on EvaGreen fluorescent dye to simultaneously detect and distinguish PEDV-nucleoprotein (N), TGEV-spike glycoprotein (S), PRV-A, PRV-C, and PCV2, and the limits of detection ranged from 5 to 50 copies/μl (Wen et al. 2019). There was no multiplex PCR method exclusively for differential detection of the four enteric CoVs until Huang et al. developed a TaqMan-probe-based real-time RT-PCR method in 2019 targeting the M gene of PEDV, the N gene of TGEV, the M gene of PDCoV, and the N gene of SADS-CoV. Their multiplex real-time RT-qPCR assay could detect 10–100 copies of each target gene per pathogen (Huang et al. 2019).

Conclusion and perspectives

As mentioned above, nested RT-PCR, RT-RPA, nanoparticle-assisted PCR, RT-LAMP, CPA-NAST, SYBR green-based real-time PCR, EvaGreen-based real-time PCR, and TaqMan probe-based real-time PCR represent single or multiplex PCR methods that have been developed to detect one, two, or four enteric pathogenic CoVs. In the field, the causative agents of swine enteric diarrhea are mixed; single RT-PCR methods are not suitable for rapid and efficient detection of CoVs even though they have higher sensitivity than multiplex RT-PCR. In addition, the PCR fragments of some single RT-PCR methods have to be subjected to agarose gel analysis to determine results, which is time-consuming. In particular, nested RT-PCR requires two PCR steps, resulting in an increased likelihood of contamination, so this method has not been widely used for pathogen detection.

For rapid and efficient detection of pathogenic swine enteric CoVs, multiplex PCR methods, including conventional multiplex RT-PCR and multiplex real-time RT-PCR, are ideal options. Although conventional multiplex RT-PCR can simultaneously differentially detect several different pathogens in one reaction, the method also possesses the disadvantages of single RT-PCR, e.g., risk of product contamination, and the inability to monitor developments in real time. The sensitivity of RT-PCR is 10–100 times lower than that of real-time RT-PCR, and the viral loads cannot be measured (Keyaerts et al. 2006). Recently, real-time TaqMan probe-based RT-PCR methods have become increasingly used to detect targets because they own many advantageous characteristics: the ability to perform differential detection, high specificity, high sensitivity, high-throughput ability, high repeatability, quantification ability, and the ability to assess results in real time (Slavov et al. 2016; Teng et al. 2015; Zhu et al. 2016a). Therefore, Huang et al. in our lab developed a TaqMan-probe-based real-

time RT-PCR for the differential detection of PEDV, TGEV, PDCoV, and PEAV (Huang et al. 2019).

Although real-time TaqMan probe-based RT-PCR possesses many merits and can be used to rapidly and efficiently detect pathogenic swine enteric CoVs, the method requires a high-precision and sophisticated instrument, practical technicians, and a good laboratory; therefore, it cannot be used for detection in under-equipped laboratories or on-site. Rapid, accurate, and more practical detection methods are of great significance for the surveillance, prevention, and control of enteric diseases in pigs, so novel assays are still deserved further development. For instance, test strip detection methods, which can be used by under-equipped laboratories or on-site and can be easily operated to quickly generate results, are urgently needed.

As mentioned earlier, apart from the four enteric CoVs, many other pathogens causing diarrhea in pigs have been identified. And the causative agents of swine enteric diarrhea are mixed in the field. To rapidly determine whether the pathogens are enteric CoVs, pan-CoV PCR is the best option to initially detect from clinical samples and followed by specific primers targeting individual swine enteric coronavirus for further identification when the result from pan-CoV PCR is positive. However, when the pan-CoV PCR results are negative, specific primers targeting other enteric diarrheal viruses are required to determine the causative agents.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no competing interests.

Ethical statement No ethical approval was required as this is a review article with no original research data.

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