



Duplex reverse transcriptase PCR for concurrent detection of porcine astrovirus and porcine kobuvirus

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ABSTRACT

Pig farming plays a vital role in economic growth. Unfortunately, the swine farming sector of India has experienced a decline, partially due to several viral and bacterial infections. These diseases have direct impact on animal health, productivity, mortality rates, and economic returns. Viral gastroenteritis, caused by enteric viruses, is a severe condition that primarily results in infection in neonatal animals. Many enteric viruses are responsible for viral gastroenteritis amongst which Porcine astrovirus (PAstV) and Porcine kobuvirus (PKV) are the emerging ones. Current diagnostic approaches are serological tests, conventional PCR, and other molecular diagnostics, which are reliable but take time and are expensive for high throughput screening. To overcome these challenges a duplex RT-PCR assay was developed for identification of PAstV and PKV in pigs to boost up the intensity of diagnosis. This approach allowed rapid and inexpensive testing, which is particularly useful for the swine population. A duplex PCR assay showed sensitivity to a reliable range of concentrations for PAstV at an LOD of 2.74 ng and for PKV at 30 fg. This developed assay also found to be specific and did not detect other porcine viruses like PCV and PPV. The applicability of assay was also done for screening (n=50) pig samples collected from different regions of Punjab, India, and the developed nucleo-diagnostic identified both viruses (PAstV and PKV) at a single stance and thus enhanced control measures that would reduce losses occasioned by these diseases in the swine industry.

Keywords: Diarrhoea, Duplex PCR, Gastroenteric, Porcine Kobuvirus, Porcine Astrovirus

The pig farming sector of India accounts for about 1.7% of all livestock population of India. This sector heavily accounts for the nation's economic development and is a means of subsistence. India has one of the highest proportions of livestock wealth. According to the 2019 census, the pig farming industry, in the country is 9.06 million in the current census and has declined by 12.03% over the previous census. The piggery sector has faced significant challenges due to the persistent onslaught of viral and bacterial infections. Infections such as Porcine Reproductive and Respiratory Syndrome (PRRS), Porcine Circovirus Associated Disease (PCVAD), Classical Swine Fever (CSF), African Swine Fever (ASF), Swine Influenza, Porcine Epidemic Diarrhoea (PED), Foot-and-Mouth Disease (FMD) and viral gastroenteritis have cast a shadow over the growth prospects of the sector (Meng *et al.* 2012). These diseases not only affect animal well-being, but also economic viability, as they induce decreased productivity, increased mortality rates, and substantial financial setbacks

for both farmers and stakeholders (Zhang *et al.* 2019). Among these diseases, viral gastroenteritis is particularly severe in young pigs, especially those under one month old, due to its rapid spread through enteric viruses via the oral-faecal route (Chauhan & Gordon, (2020). Symptoms such as diarrhoea, vomiting, dehydration, and fever can lead to a high mortality rate of up to 52%, as reported by the National Animal Health Monitoring System in 2011. Various enteric viruses, including Porcine astrovirus (PAstV) and Porcine kobuvirus (PKV), are the primary causes of viral gastroenteritis in piglets. These viruses can replicate within the swine population and cause explosive outbreaks. The northern part of India has a high population density of swine making it a perfect place for these enteric viruses to circulate. Most of these diseases tend to occur during winter season stating that seasonality also plays a significant role during these viral infections. PAstV and PKV are two emerging RNA viruses that are being identified more frequently as crucial agents in swine, mainly due to their relationship with gastrointestinal diseases and their ability to exacerbate illness, particularly causing mild to severe diarrhoea. PAstV belongs to the family *Astroviridae* and the subfamily *Mamastrovirus* and is a small, non-encapsulated virus, with a single positive strand RNA genome (Kour *et al.* 2021). Another similar virus is part of the *Picornaviridae* family and the *Kobuvirus*

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genus which is also a non-enveloped virus, which affects the Gastrointestinal system. Not only in India but these two viruses have shown worldwide distribution with the different genotypes reported in Europe, North America, and Asia including India (Rodriguez and Arzt 2022).

Early detection and precise identification of these PAsV and PKV is critical in control of an epidemic (Khamrin *et al.* 2010). The early identification of these viruses has also been enhanced by some molecular diagnostics like conventional PCR, electron microscopy and sequencing. However, these molecular diagnostics pose several challenges during the identification and screening of viruses such as more time-consuming and less sensitive (Mustafa *et al.* 2020). These limitations make these methods less suitable for use in large-scale and rapid diagnosis of diseases (Abdeldayem *et al.* 2022). Therefore, it is important to design a nucleodiagnosics like duplex RT-PCR assay that aims at the simultaneous identification of PAsV and PKV at the same time, which not only increases the specificity of the assay but also reduces the time and cost of the test. The development of the assay aims to improve disease diagnosis by simultaneously detecting both viruses, thereby expediting disease management.

MATERIALS AND METHODS

Primer designing: During the development of duplex PCR for PAsV and PKV, primers used for PAsV amplification (Kumthip *et al.* 2018) whereas for PKV primers, were synthesized by doing the multiple sequence alignment of 5'-UTR gene of PKV with the complete domain sequences targeting 5'-UTR region of PKV sequences of India, a conserved sequence for the gene. Primers for PKV were synthesized in-house using Primer 3 and Primer-BLAST.

Sequence retrieval and alignment: From the NCBI GenBank database, total of seven full-length sequences (complete domain sequences) of 5'-UTR regions of PKV from the Indian isolates were gathered, which further was subjected to sequence alignment to know the conserved region between all sequences. Details of retrieved sequences are given in Supplementary Table 1. For the alignments of the obtained sequences, an alignment tool, BioEdit, was used. The conserved region, hence, was used further for the in-house synthesis of primers of PKV in duplex PCR. Details of primers used during the development of the assay are given in Table 1.

Duplex PCR standardisation: The duplex PCR was standardized using both sets of primers together in a

single reaction tube (Table 1). Briefly, 0.5 picomolar final concentration of each of primers (forward and reverse) for both PAsV and PKV were used in a single reaction initially (uniplex). The reaction mixture components as well as the amplification protocol 95°C for 2 min, 95°C for 1 min followed by an annealing temperature of 53°C for 25 sec, an extension of 72°C for 1 min for 35 cycles, and a final extension of 72°C for 10 min. The final annealing temperature of duplex PCR was standardized by performing gradient PCR of self-synthesized primers for PKV. The reaction was carried out on the Mastercycler Nexus Gradient (Eppendorf, Germany). The PCR reaction mixture had a total volume of 20 µL, comprising 10 µL of 2X DreamTaq Master Mix (final concentration 1X), 1 µL of each forward and reverse primer for PAsV and PKV (10 picomoles/µL, final concentration), and 2 µL of the cDNA template and rest volume was makeup by adding 4 µL NFW. To ensure specificity and avoid non-specific amplification, a non-template control (NTC) was included in each reaction set. The NTC, containing all reaction components except the cDNA template, helps detect any contamination or primer-dimer formation. During the standardization of the duplex PCR assay, known positive cDNA for PKV and PAsV were utilized

Sensitivity: The analytical sensitivity of the duplex PCR was carried out for PAsV and PKV separately. Briefly, the cDNA was serially diluted by 10-fold dilution ranging from 2741 nanogram/µL to 2.74 femtogram/µL for PAsV and 3057 ng/µL to 305 ag/µL for PKV. The PCR reaction was carried out using the optimized reaction conditions and amplification protocol

Specificity: The specificity of the developed duplex PCR assay was carried out using PAsV and PKV cDNA as well as with the DNA of other viruses. These included DNA from viral pathogens such as Porcine parvovirus2 (PPV2) and Porcine circovirus 2 (PCV2). The PCR reaction was carried out using the optimized reaction conditions and amplification protocol.

Applicability of duplex PCR with field samples: Fifty faecal samples (n=50) with known positives and negatives for PAsV and PKV were taken to evaluate the applicability of the developed assay. (Supplementary Table 2). RNA was extracted from faecal samples of pigs which was further used to synthesize cDNA, the synthesized cDNA was further used as a template for screening of PAsV and PKV. The concentrations of cDNA of all the known positive and negative samples to be used as templates were brought to a similar range by dilution. Duplex PCR was performed

Table 1. Self-Designed and Published Primers for Nucleo-Diagnostic development for duplex PCR

Sl. No.	Primer Name	Sequence (5' – 3')	References
1	PAstV-F	TGACATTTTGTGGATTACAGTT	Kumthip <i>et al.</i> 2018
2	PAstV-R	CACCCAGGGCTGACCA	
3	PKoV-5'UTR-F	GCTACCAAACCTCCTACCCGA	In-house synthesized
4	PKoV-5'UTR-R	AGTACATGGTCAAGCGTGGA	

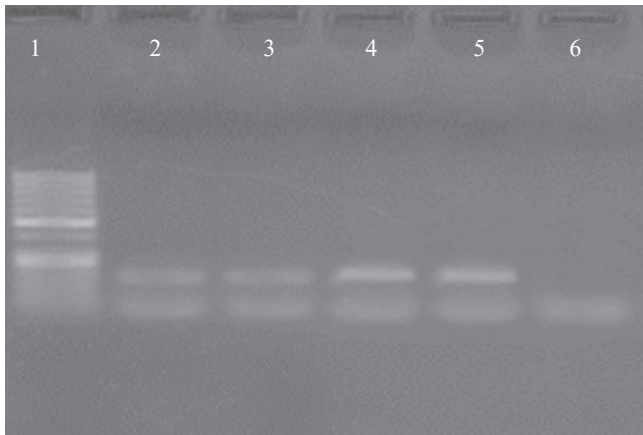


Fig. 1 Agarose gel electrophoresis showing the gradient PCR of PKV primers at 53°C, 55°C, 57°C, and 59°C
Lane1: 100 bp ladder; Lane2: Amplified 220 bp product at region 59°C; Lane3: Amplified 220 bp product at region 57°C; Lane 4: Amplified 220 bp product at region 55°C; Lane 5: Amplified 220 bp product at region 53°C; Lane 6: NTC

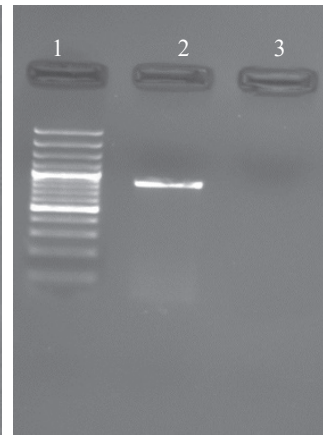


Fig. 2 Agarose gel electrophoresis showing the amplified product at 799 bp of RdRp gene of PAsV
Lane1: 100 bp ladder; Lane2: Amplified 799 bp region; Lane3: NTC

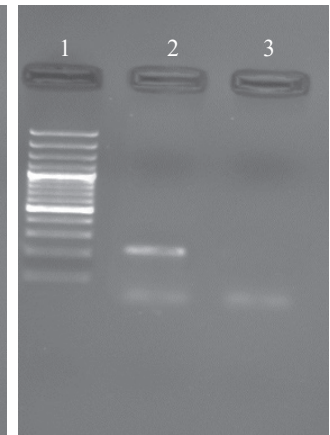


Fig. 3 Agarose gel electrophoresis showing the amplified product at 220 bp of the 5'-UTR gene of PKV
Lane1: 100 bp ladder; Lane2: Amplified 220 bp region; Lane3: NTC

on all the samples. The PCR product was used as positive control and nuclease-free water (NFW) was added in place of template cDNA in non-template control. The reaction composition and conditions were followed as of standardized duplex PCR.

RESULTS AND DISCUSSION

Duplex PCR enables simultaneous amplification of two distinct DNA targets in one reaction, enhancing efficiency and reducing reagent cost (Deng *et al.* 2016). This technique is particularly useful in virology for detecting co-infections among enteric pathogens in livestock, supporting effective disease management (Kumar *et al.* 2020). This method not only reduces the time and cost associated with testing but also improves the ability to identify co-infections at once, which is crucial for understanding the epidemiology and management of viral diseases in swine populations. Co-infections with multiple viruses can lead to synergistic effects, increasing pathogenicity or altering the clinical presentation of disease. So, using duplex PCR to detect both PAsV and PKV helps in giving a complete information that can help with targeted interventions, better management, and the developments of vaccines and treatments. This streamlined approach is particularly useful in diagnostics where rapid results are crucial, such as in managing outbreaks or in routine surveillance of viral pathogens in swine populations.

In this study, a duplex PCR assay was developed to detect both PAsV and PKV simultaneously. We used specific primers for each virus, with PAsV primers targeting the RdRp region to generate a 799 bp amplicon, and PKV primers amplifying the 5'-UTR regions of the virus genome to generate a 220 bp amplicon. The primer set for PAsV had an annealing temperature at 53 °C. To develop a duplex PCR for both PAsV and PKV, it became necessary to find a common annealing temperature suitable

for both sets of primers. For the self-synthesized PKV primers, gradient PCR was performed at four temperatures: 53°C, 55°C, 57°C, and 59°C using a positive control (Fig. 1). The results established satisfactory amplification for PKV at 53 °C, which is in harmony with the annealing temperature for PAsV. Molecular detection of the specific genome regions of PAsV and PKV was also carried out using uniplex PCR. For both viruses, in a PCR reaction, 1µl of each primer was used at annealing temperature of 53°C and achieved satisfactory amplification. Therefore, we set the annealing temperature of the duplex PCR at 53°C to facilitate the amplification of both viruses. The same concentration of primers was used during duplex PCR. The amplifications were performed in a PCR thermal cycler (Eppendorf, Germany) using DreamTaq® Green Master Mix (2X). The cycling conditions were an initial denaturation at 95°C for 2 minutes, followed by 35 cycles of 95°C for 1 minute, 53°C for 25 seconds, and 72°C for 1 minute, with a final extension at 72°C for 10 minutes for both PAsV and PKV. Uniplex PCR for PKV, which targets a 220 bp region, a specific product was observed on agarose gel electrophoresis Fig 2.

Similarly, we detected a specific 799 bp product for PAsV, which targets the RNA polymerase gene (Fig. 3). The duplex PCR was then standardized by combining both sets of primers in a single reaction tube. During the optimization of the duplex PCR, the final concentration of each primer was set to 1 µM, and a uniform annealing temperature of 53°C was used for both targets (Fig. 4). The study by Zhang *et al.* (2019) developed a duplex PCR assay for the simultaneous detection of Porcine Astrovirus and Porcine Kobuvirus, enhancing diagnostic efficiency. Xu *et al.* (2012) demonstrated a multiplex PCR method for the simultaneous detection of multiple porcine viruses, improving diagnostic capabilities.

Sensitivity of duplex PCR (PAsV and PKV): The

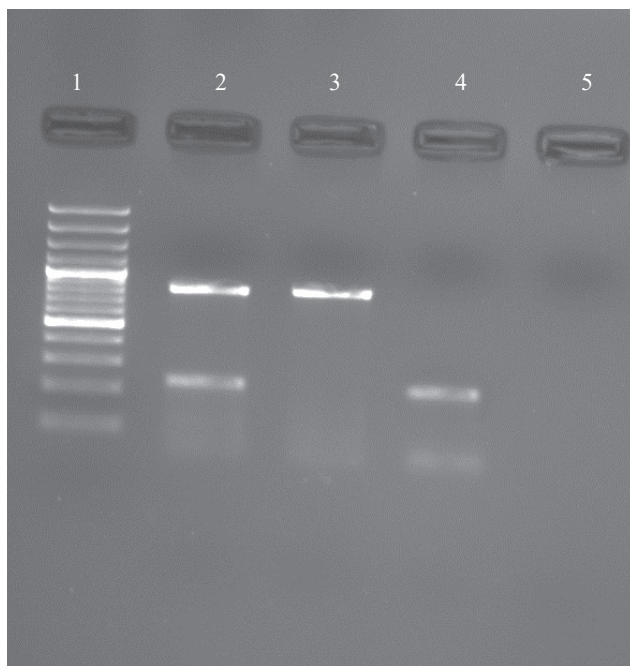


Fig. 4 Duplex PCR for PAsTV and PKV Specificity Assay
Lane 1: 100 bp ladder; Lane 2: (DUPLIX of PAsTV and PKV) (53°C); Lane 3: Positive Control PAsTV; Lane 4: Positive Control PKV; Lane 5: NTC

sensitivity of the duplex PCR assay for detecting PAsTV and PKV was evaluated through a series of experiments involving serial dilutions of known positive samples. For PAsTV, a dilution series was prepared ranging from 2741 ng/μL down to 2.74 fg/μL. Similarly, for PKV, a dilution series was prepared ranging from 3057 ng/μL down to 305 ag/μL. These dilutions were carefully made to cover a wide range of viral concentrations, enabling a thorough assessment of the sensitivity of the developed assay. Following amplification, the sensitivity PCR products were analyzed using Agarose gel electrophoresis with 1.5% agarose gel. The resulting gel images (see Fig. 5 and Fig. 6) displayed distinct bands corresponding to the targeted viruses, demonstrating the assay's effectiveness in amplifying the viral cDNA to a certain range.

The limit of detection (LOD) for PAsTV was found to be 2.74 ng/μL (Fig. 5), while the LOD for PKV was 30 fg/μL (Fig. 6). At these specific concentrations, consistent amplification of the targeted amplicons was observed till a certain range of concentration, further confirming the assay's high sensitivity. These results underscored the reliability of the duplex PCR method in detecting low viral loads. One study (Zhang *et al.* 2023) also developed a multiplex PCR for detecting and differentiating five PAsTV genotypes simultaneously demonstrated high sensitivity and specificity. The assay successfully identified PAsTV in 47.4% of pig fecal samples, showing a >96% coincidence rate with traditional monoplex PCR methods.

Specificity of Duplex PCR (PAsTV and PKV): The specificity of the developed duplex PCR assay was tested by running the duplex reaction with other homologous

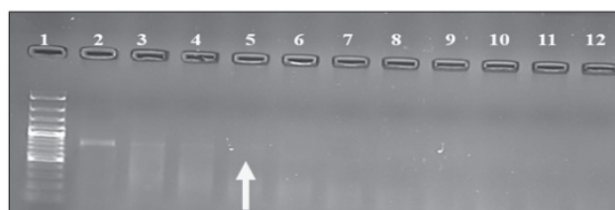


Fig. 5 Sensitivity Assay of Primer (PAsTV)
Lane 1: 100 bp DNA Ladder; Lane 2: 2741 ng/ul; Lane 3: 274.1 ng/μl; Lane 4: 27.4 ng/ul; Lane 5: 2.74 ng/ul; Lane 6: 274 pg /ul; Lane 7: 27 pg /ul; Lane 8: 2.74 pg /ul; Lane 9: 274 fg /ul; Lane 10: 27.4 fg/ul; Lane 11: 2.74 Fg /ul; Lane 12: NTC
Amplification was observed up to lane 5 i.e., 2.74 ng/μl of genomic DNA template

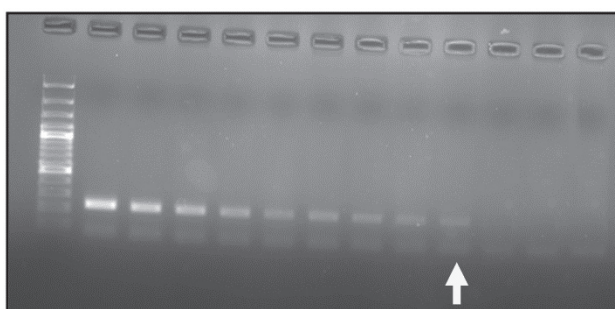


Fig. 6 Sensitivity Assay of Primers (PKV)
Lane 1: 100 bp DNA Ladder; Lane 2: 3057 ng/ul; Lane 3: 305.7 ng/μl; Lane 4: 30.5 ng /ul; Lane 5: 3.05 ng /μl; Lane 6: 305 pg /ul; Lane 7: 30 pg /ul; Lane 8: 3.05 pg /ul; Lane 9: 305 fg/ul; Lane 10: 30.5 fg /ul; Lane 11: 3.05 fg/ul; Lane 12: 305 ag; Lane 13: NTC
Amplification was observed up to lane 5 i.e., 2.74 ng/μl of genomic DNA template

host viruses, specifically Porcine Parvovirus 2 (PPV2) and Porcine Circovirus 2 (PCV2). When the products were run on 1.5% gel, no amplifications were observed in the PCV2 and PPV2 samples (Fig. 7), indicating that the duplex PCR assay is highly specific for PAsTV and PKV, with no cross-reactivity with these other viruses.

Duplex PCR on samples (Applicability of Duplex PCR): The performance of the assay was determined using 50 samples (known positive and negative) based on their PAsTV and PKV status which included positive(n=10) and

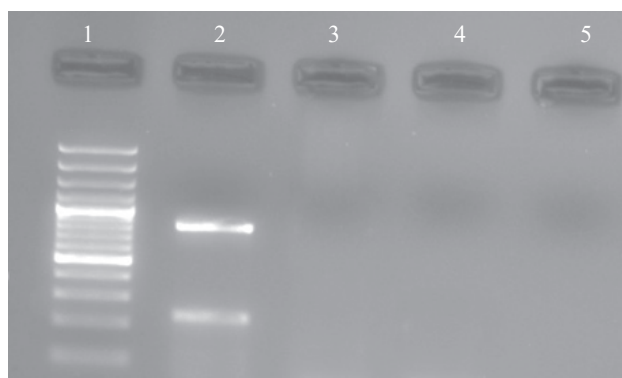


Fig. 7 Specificity Assay of duplex PCR (PAsTV and PKV)
Lane 1: 100 bp ladder; Lane 2: (DUPLIX); Lane 3: PPV 2; Lane 4: PCV 2; Lane 5: NTC



Fig. 8(a) Assessment of applicability of duplex PCR.
(PAsTV and PKV)

Lane 1: 100 bp ladder; Lane 2- Lane 20: P1 to P19; Lane 21: 100bp ladder; Lane 22- Lane 40: P20 to P37 field samples; Lane 40: NTC

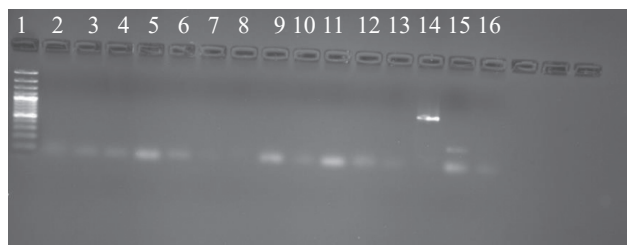


Fig. 8(b) Assessment of applicability of duplex PCR.
(PAsTV and PKV)

Lane 1: 100 bp ladder; Lane 2- Lane 19: P38 to P50; Lane 14: Positive Control for PAsTV; Lane 15: Positive Control for PKV; Lane 16: NTC

negative ($n=40$) samples for PAsTV, respectively. Whereas, known 21 positive animals were PKV positive, and 29 samples had negative results for PKV. Seven samples were positive for both PKV and PAsTV. Each sample was subjected to the duplex PCR assay under standardized conditions. In this study, 10 samples tested positive for PAsTV, and 21 samples tested positive for PKV (Fig. 8 (a) and (b)). Seven samples were co-infected with both PAsTV and PKV, demonstrating the presence of both enteric virus (PAsTV and PKV) infections in faecal samples. This study successfully developed a duplex PCR assay that gave the same positive and negative results for both PAsTV and PKV in samples that were known to be positive or negative in monoplex method. This showed that duplex PCR is a reliable way to detect PAsTV and PKV simultaneously. Further, in the testing, 7 samples were found positive for both PAsTV and PKV showing co-infection, meaning the presence of two viruses altogether. The successful amplification of viral targets in these samples indicates that the duplex PCR assay is reliable for monitoring and managing viral infections in swine populations across various geographic locations. The study by Xiao *et al.* 2023 identifies multiple novel porcine astroviruses (PAsTV) and highlights a high prevalence of co-infection among pigs, with individual animals frequently harboring multiple PAsTV types simultaneously.

This co-infection is suggestive of a complex viral ecology in swine populations, contributing to the diversity of PAsTV strains. Another report by Ouyang *et al.* (2019) discusses the co-infection of swine with porcine circovirus type 2 (PCV2) alongside other swine viruses, noting that such co-infections are common and can exacerbate the clinical severity of the disease.

The results of the applicability of the developed technique indicated that for some samples, both PAsTV and PKV are detected in the duplex PCR which indicated the presence of co-infection (e.g., Samples P1, P2, P4, P6, P7, P10, and P22). Overall, duplex PCR provides more comprehensive information by detecting both viruses simultaneously, whereas conventional PCR focuses solely on detection of a single virus at a moment. One study by Deng *et al.* (2020) showed the development of a multiplex RT-PCR assay aimed at simultaneously detecting multiple major diarrheal viruses in pig herds. This assay was designed to improve diagnostic capabilities and streamline the detection process for these viruses, which is critical for effective disease management in swine. Relating the study by Ding *et al.* 2020 to our duplex PCR for PAsTV and PKV, the study highlights the advantages of using multiplex PCR techniques for comprehensive virus detection in livestock. Similar to Ding *et al.*'s approach, our duplex PCR assay allows for the simultaneous detection of two viruses PAsTV and PKV using a single test. This parallel approach enhances efficiency, reduces the need for multiple tests, and improves the accuracy of diagnostics, which is crucial for monitoring and controlling viral infections in pigs. An additional study by Capai *et al.* (2022) focuses on the detection of various porcine enteric viruses, including Kobuvirus, Mamastrovirus, and Sapleovirus, in domestic pigs in Corsica, France. This is relevant to our duplex PCR for PAsTV and PKV as it underscores the importance of developing targeted assays for detecting specific viruses in pigs, aiming for comprehensive viral surveillance and improved diagnostic accuracy.

The present study, which describes the development and standardization of a duplex PCR assay for the simultaneous detection of PAsTV and PKV, has the potential to be a valuable diagnostic method. This creates a single reaction that lowers the risk of contamination, speeds up diagnosis on co-infected animals, uses less time and reagents, and helps with the effective management of affected swine populations. The developed assay showed high sensitivity and specificity, which was equivalent to conventional individual PCR assays for PKV and PAsTV. The developed Duplex PCR assay is a significant improvement in diagnostic tools for enteric viruses in livestock in general and in pigs in particular, as it aligns with efforts to improve viral surveillance and disease control measures in the swine industry.

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