SUPPLEMENTARY MATERIAL AVAILABLE ONLINE

Indian Journal of Animal Sciences **95** (11): 985–990, November 2025/Article
https://doi.org/10.56093/ijans.v95i11.166592

Nested polymerase chain reaction assay for detection of Porcine Astrovirus in pig population

AAKRITI PATHANIA[1], ADARSH MISHRA[1], VISHAL MAHAJAN[2] and YASHPAL SINGH MALIK[1]✉

Guru Angad Dev Veterinary and Animal Sciences University, Ludhiana, Punjab-141 004, India

Received: 14 May 2025; Accepted: 06 February 2026

ABSTRACT

Porcine Astrovirus (PAstV) is a positive-sense single-stranded RNA virus, responsible for gastrointestinal diseases in swine populations across the globe. There are sparse reports regarding diagnostic platforms for the detection of PAstV in the porcine population. The present study reported a nested PCR (nPCR) assay for the identification of PAstV in faecal specimens of pigs in Punjab, India. The technique was developed using outer and inner sets of primers targeting the RNA-dependent RNA polymerase (RdRp) gene of the virus. The nPCR was standardized and optimized, including sensitivity and specificity. The sensitivity of the assay was found to be 225 femtograms. The technique was specific and did not amplify with certain other viruses *i.e.,* Porcine Kobuvirus (PKV) and Bovine Rotavirus (BoRV). The applicability of the nPCR assay was further assessed on faecal samples (n=50) collected from field conditions comprising diarrhoeic (n=39) and non-diarrhoeic (n=11) animals. The nPCR detected only 12 [diarrhoeic (n=8) & non-diarrhoeic (n=4)] out of 50 samples tested as positive. This work demonstrated that nPCR assay can be effectively used as a rapid, specific, and sensitive method for routine molecular screening and/or diagnosis of PAstV in swine population.

Keywords: Astrovirus, Gastroenteritis, Nested PCR, Porcine, Sensitivity, Specificity

Global food security is one of the prime concerns worldwide.. India is one of the agrarian countries of the world. While agriculture makes up 4.2% of India's Gross domestic product (GDP), the livestock sector contributes a considerable 25.6% of the country's social and economic growth (Opriessni *et al.* 2020). The livestock sector has an annual growth rate of 7% over the past three decades, more than twice the growth of the agricultural sector. Various viral and bacterial diseases have affected the potential of the livestock sector. Viral gastroenteritis is one serious issue that frequently affects farm animals. It is caused by enteric viruses and mainly leads to symptoms like diarrhoea, nausea, and vomiting (Puente *et al.* 2023). Among the list of enteric viruses, Astrovirus is a non-enveloped, positive-sense single-stranded RNA virus, belonging to the family *Astroviridae* (Fang *et al.* 2019). The members of *Mamastrovirus* genus of *Astroviridae* family infect humans, pigs, cattle, and other mammals, which also include Porcine Astrovirus (PAstV) (Guix *et al.* 2013). PAstV is a serious viral pathogen that affects swine populations worldwide (Rawal and Linhare, 2022). Although PAstV has been associated with enteric diseases, it has also been occasionally implicated in certain severe cases with neurological symptoms (Kumthip *et al.* 2018). Although generally associated with diarrhoea-causing gastroenteritis, astroviruses have recently been found associated with neurological diseases in pigs which highlights their detrimental effects on animal health (Zhang *et al.* 2023). The virus is highly mutable and might produce new strains that may hinder attempts at control. Various factors make it difficult to detect and diagnose PAstV in pigs which include the genetic diversity of the virus and the subclinical infections (Staubli *et al.* 2021). Furthermore, the virus is frequently associated with mild or asymptomatic infections that tend to be underdiagnosed. The virus cultivation is time-consuming whereas serological tests on the other hand require a high sensitivity level to detect early stages of infection. Thus, conventional virus cultivation as well as serology-based assays make detection of the virus more challenging particularly in cases where the diseased animal is in the early stages of infection or is a carrier showing no signs of infection. Molecular diagnostics are better alternatives for a specific and accurate diagnosis of the pathogen. The nested PCR (nPCR) is useful for the identification of PAstV because of the high sensitivity that enables to detection of the virus even in cases of viral infection with low antigenic load. The present study has developed a highly sensitive and specific nPCR assay for the detection of PAstV.

Present address: [1]College of Animal Biotechnology, Guru Angad Dev Veterinary and Animal Sciences University, Ludhiana, Punjab, India. [2]Animal Disease Research Centre, College of Veterinary Science, Guru Angad Dev Veterinary and Animal Sciences University, Ludhiana, Punjab, India. ✉Corresponding author email: malikyps@gmail.com

MATERIALS AND METHODS

Sample collection and processing: Faecal samples (n=50) were collected from pigs, with cases of diarrhoea suspected of gastrointestinal infection including both diarrhoeic (n=39) and non-diarrhoeic (n=11) animals from various organized and unorganized farms in Punjab. The samples were transported to the laboratory over ice. The collection and transportation of the clinical samples were done with complete adherence to biosafety precautions. Freshly collected faecal samples were processed and a 10% (w/v) suspension was prepared in phosphate-buffered saline (PBS, pH 7.2). The samples were stored at -80°C until they were subjected to further analyses.

RNA extraction: Total RNA was extracted from a 600 µl clarified 10% (w/v) faecal suspension in 1 ml RNAiso (Cat# 9108, TAKARA, Japan) reagent. It was vortexed and incubated at room temperature, followed by the addition of 200 µl of chloroform. The aqueous phase was collected after a 15-minute centrifugation at 12,000 rpm at 4°C and mixed with an equal volume of isopropanol. The mixture was precipitated for 4 hours at -20°C. The RNA pellet thus obtained was again spun down, washed in ice-cold 70% ethanol, dried, and re-suspended in 20µl of RNase-free water. The yield and quality of isolated RNA were analyzed by a Nanodrop spectrophotometer (ThermoScientific, USA).

Synthesis of cDNA: A 10 µl volume of total RNA (1000 ng) was taken and mixed with 1µl of random hexamers and 1 µl nuclease-free water. It was incubated at 95°C for 5 minutes and snap-cooled on ice. To the reaction mixture, 5 µl of 5X RT buffer, 1 µl of 10 mM dNTP, 1 µl of MMLV-RT (200 U/µl), and 1 µl of Ribolock (40 U/µl) were added to make a total volume of 20 µl. This mixture was incubated at 25°C for 5 minutes, followed by incubation at 42°C for 60 minutes and finally at 72°C for 5 minutes to inactivate the reverse transcriptase. The synthesized cDNA was stored at -20°C.

Sequence retrieval and primer design: A total of fourteen PAstV RdRp gene sequences of Indian origin along with the NCBI reference sequence of the RdRp gene (Accession no. NC_023675) were retrieved from the NCBI GenBank database, aligned through BioEdit sequence alignment editor tool (Hall, 1999) and used in the subsequent analysis for primer designing (Table 1). The nPCR was developed using an outer set of primers as reported (Lambisia, 2021) and an in-house designed inner set of primers (Fig. 1 and 2). The strategy for the designing primers is as shown (Fig. 3).

Standardisation of nPCR: The first round of nPCR amplification, using outer sets of primers (similar to conventional PCR), was performed using Mastercycler Nexus Gradient (Eppendorf, Germany). The PCR reaction mixture was composed of a total volume of 20 µl consisting of 10 µl of 2X DreamTaq Master Mix (final concentration 1X), 1 µl of each outer forward and reverse primer (Stock concentration: 10 picomoles/µl; final concentration 0.5 picomoles/µl), and 2 µl of the cDNA template. A no-template control (NTC) was included in each reaction set. The amplification conditions were initially optimized by annealing temperature gradient, with increments of 2°C, ranging from 52°C to 58°C. The finalized protocol for the first round of amplification involved an initial denaturation step at 95°C for 2 minutes, denaturation at 95°C for 60 seconds followed by 30 cycles of annealing at 52°C for 30 seconds, and extension at 72°C for 60 seconds followed by final elongation step at 72°C for 10 minutes. For standardization, a 799 bp genomic region of the RdRp gene using an outer set of primers was amplified, cloned in pJET 1.2 vector, transformed in *E. coli* TOP 10 host, and sequenced using one of the suspected clinical samples collected from a highly diarrhoeic pig of 6 months of age, which was subsequently used as a positive control. The

Table 1. Details of Nucleotide Sequences retrieved in study for PAstV

Sr. No.	Accession No.	Strain/Isolate	Position	Year of collection
1.	NC_023675	PAstV 4 Strain 35/USA	75-4072	2010
2.	KX_453785	PAstV/Por-wt isolate UP-30	1-299	2014
3.	KX_453786	PAstV/Por-wt isolate ASM-123	1-240	2014
4.	KX_453787	PAstV/Por-wt isolate ASM-129	1-267	2014
5.	KX_453788	PAstV/Por-wt isolate ASM-135	1-299	2014
6.	KT_757529	PAstV/Por-wt isolate IVRI Por-30	1-315	2013
7.	KT_757530	Isolate PAStV/Por-wt/NER-ASM	1-239	2013
8.	KT_757531	Isolate PastV/Por-wt/NER-ASM	1-267	2013
9.	KT_757532	Isolate PastV/Por-wt/NER-Asm	1-299	2013
10.	KJ_650562	PAstV-NER-161P isolate Past-NER	1-284	2013
11.	KJ_650563	PAstV-NER-162P isolate Past-NER	1-285	2013
12.	KJ_650564	PAstV-NER-163P isolate Past-NER	1-282	2013
13.	KJ_650565	PAstV-NER-164P isolate Past-NER	1-285	2013
14.	KJ_650566	PAstV_NER_175P isol ate Past-NER	1-299	2013

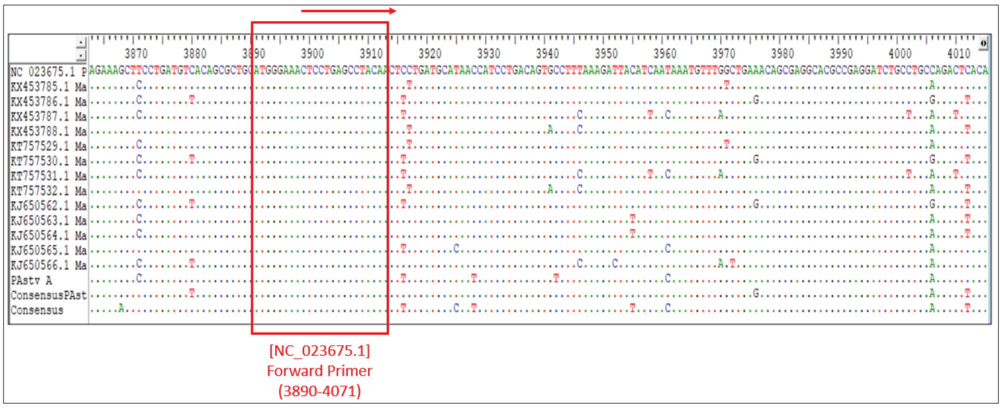

Fig. 1 Sequence alignment Conservation dot plot (PAstV Forward Primer (RdRp gene))

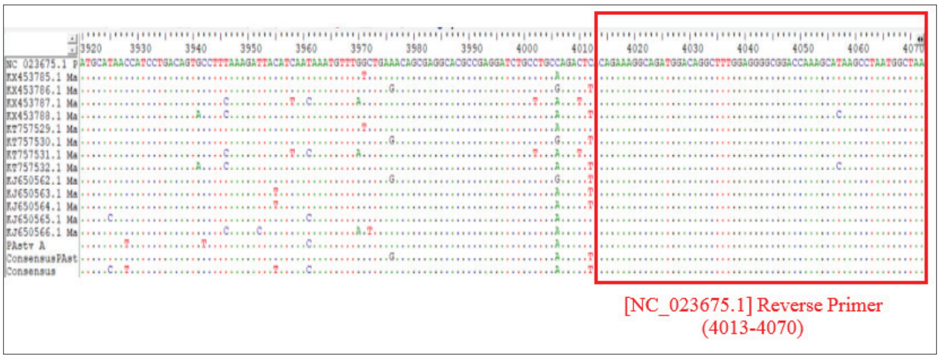

Fig. 2 Sequence alignment Conservation dot plot (PAstV Reverse Primer (RdRp gene))

amplified PCR product was separated in ethidium bromide (0.5µg/ml) stained 1.5 % agarose gels in 1X Tris-acetate EDTA (TAE) buffer (pH 8.0) at 80 volts. The GeneRuler™ 100 bp DNA Ladder was used as the molecular marker during agarose gel electrophoresis. The separated PCR products were visualized under ultraviolet (UV) light and recorded in a Gel documentation system (G Box, Syngene, UK).

Amplification with inner set of primers: Inner primers as listed in Table 2 were used for standardization of the second round of amplification, following similar amplification conditions used for the first round of amplification. Initially, during standardization, the inner primers were used directly in the genomic cDNA instead of the first round amplified PCR product using similar composition of reaction mixture; only the outer set of primers was replaced with the inner set of primers. Gradient PCR with annealing temperatures of 2°C increments, *i.e.,* 52°C, 54°C, 56°C, and 58°C, was used for standardization of an inner set of primers similar to the outer ones. The temperature depicting the sharp and specific band was chosen for standardization.

Adoption of nested reactions (nPCR): Following standardization of the first and second rounds of amplification using the published and in-house designed sets of primers respectively, with the template cDNA, the nPCR was then optimized. It was done in two consecutive cycles. The first round of PCR was done using the outer primers which yielded an amplified PCR product. This product was then used directly as the target amplification in the second round using the inner set of primers known as nested primers. To further optimize the second-round amplification, initially, a gradient PCR was carried out. This involved gradient PCR with different annealing temperatures, starting from 52°C to 58°C with an interval of 2°C. Finally, the nPCR was standardized at 52°C.

Sensitivity of nPCR: For detection of the sensitivity of the nPCR technique developed, the known PAstV positive cDNA template was serially ten-fold diluted *i.e.* from 2254 nanogram/µl to 2.2 attogram/µl and tested for positivity. The identical reaction components and standardized

Table 2. Primers used for Nested PCR

Sl. No.	Primer Name	Sequence (5´-3´)	Amplification Product size	REFERENCES
1	Outer-F	TGACATTTTGTGGATTTACAGTT	799 bp	[9]
2	Outer-R	CACCCAGGGCTGACCA		
3	Inner-F	CATGGGAAACTCCTGAGC	182 bp	Designed in the study
4	Inner-R	TAGCCATTAGGCTTATGC		

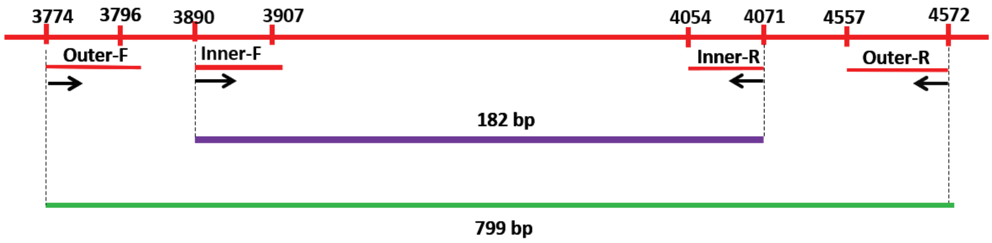

Fig. 3 Nested PCR conditions of PAstV

amplification settings were used.

Specificity of nPCR: The specificity of the nPCR for PAstV detection was evaluated using known positive cDNA samples of other viruses, *i.e.*, viruses of homologous host [Porcine Kobuvirus (PKV)] and one heterologous host [Bovine Rotavirus (BoRV)]. Genomic templates of PKV and BoRV were tested negative for the nPCR assay.

Applicability of nPCR with field samples: To check the applicability of the developed assay suspected field samples (n=50) were collected from diarrhoeic as well as non-diarrhoeic pigs and these samples were screened for the presence of targeted virus in field samples using the developed technique. The concentrations of cDNA of all the clinical samples to be used as templates were brought to a similar concentration before screening.

RESULTS AND DISCUSSION

Viral gastroenteritis in farms is a big issue as it affects the health of animals and productivity. PAstV is one of the main causes of viral gastroenteritis in pigs of different ages: piglets and adult swine (Piryaei *et al.* 2023). The virus leads to severe watery diarrhoea, vomiting, and dehydration, which results in weight loss, slow growth rates, and can even be fatal in certain cases (Li *et al.* 2023). In farms, viral gastroenteritis can be easily transmitted among the animals by contact and cause lots of havoc under circumstances such as crowdedness and poor hygiene. Minimizing the effects of such viruses and ensuring proper health and production rates of the animals requires proper management practices and biosecurity measures to be put in place. However, today's diagnostic approaches in detecting and screening PAstV in farm animals have progressed from basic to advanced. With advancements in technology, the Enzyme-Linked Immunosorbent Assay (ELISA) was widely used since it was able to identify viral proteins, but it was sometimes not sensitive, especially in cases of early infections and required specific antibodies (Salamunova *et al.* 2018). So, the advent of nPCR, brought further enhancement in the testing of enteric viruses as it made it possible to detect viral RNA with high sensitivity and specificity (Meena & Singh, 2013). In this study, the optimized nPCR using Taq polymerase PCR conditions was designed to detect PAstV up to a certain range of concentration of the virus.

Standardisation: For the initial standardisation, the nucleo-diagnostic gradient PCRs were used to normalize the amplification of the PAstV genome, employing distinct primer sets for the outer and inner regions (Supplementary Fig. 1 and 2) of 799 bp and 182 bp genomic targets respectively. For both sets of primers, an annealing temperature of 52°C was chosen so that they could be utilized in subsequent nPCR reactions for nesting. The nPCR was standardized using gradient PCR with both sets of primers altogether. After a gradient PCR (Supplementary Fig. 3), 52°C temperature is found to be the best suited for the nPCR.

Sensitivity: To evaluate the sensitivity of nPCR, the lowest dilution in the 10-fold series that resulted in amplification was identified as the minimal detection limit. For the first set of primers exclusively in conventional PCR (Supplementary Fig. 4 (a)), the minimal detection limit was found to be 22.5 nanogram/µl of genomic cDNA template, whereas for nPCR (Supplementary Fig. 4 (b)), it was 225 femtogram/µl. The findings made it evident that layering of reactions increased the assay's sensitivity, thereby making it more efficient in detecting the virus in diarrhoeic and non-diarrhoeic field samples. This development has aided in creating better strategies to identify PAstV and control the disease in the livestock field when it occurs. When differentiating nPCR assays in detecting PAstV from other conventional PCR methods, some advantages come with them (Li *et al.* 2023). In contrast to conventional PCR, nPCR has two cycles of amplification, which allows for a high detection limit; and since low viral quantities are easily attained in faecal samples, nPCR is ideal for such samples (Foo *et al.* 2020). Although there are benefits to applying this technique to quantify PAstV and generate data more rapidly, the procedure has been found to have low sensitivity, particularly when concentrations of viral RNA are low (Jalal *et al.* 2021).

Specificity: The published and self-designed primer sets used during nPCR specificity particularly recognized the RdRp gene of the PAstV genome, and no cross-amplification was observed for the other homologous and heterologous host viruses investigated during the study, i.e., PKV and BoRV (Supplementary Fig. 5 (a) and (b)). The study by Caruana *et al.* (2020) also stated the advantages of specificity of nPCR, especially in the context of field samples with potentially low viral titer. Another finding by Bae *et al.* (2022) mentioned that the nested RT-PCR assay was very specific for amplifying hepatitis A and E viral RNA only and did not cross-react with other pathogens.

Furthermore, one of the specificity advantages provided by nPCR is its suitability for dealing with field fecal samples, which often contain more than one pathogen or different types of contaminants that can influence the outcomes of less specific diagnostic tools (Wildi & Seuberlich, 2021).

Applicability of nested PCR on field samples: For testing applicability of the nucleo-diagnostic developed so far, its applicability was tested in field samples collected. Initially, 50 suspected clinical samples collected from various districts of Punjab, India, were screened for PAstV by using conventional PCR with the outer set of primers. After the successful first round of amplification, the second round of amplification in the nPCR was performed using the inner primers on all 50 field samples. On comparative analysis initially, 10 samples tested positive with the outer primer set (Conventional PCR) [(Supplementary Fig. 6 (a) and (b)], while, 12 samples were identified as positive while the remaining samples tested negative, with two additional samples that came out positive [(Supplementary Fig. 7 (a) & (b)]. This indicated that nested PCR improved both specificity and sensitivity, offering a more reliable confirmation of PAstV genome compared to conventional PCR alone. This result highlights the superior sensitivity of the nested PCR method for detecting PAstV, demonstrating its ability to identify the virus even at very low antigenic titers. The enhanced sensitivity is crucial for accurate diagnosis and effective monitoring of PAstV in swine populations, ensuring that even a minimal viral presence is detected (Table 3).

Thus nested PCR is one reliable nucleo-diagnostics for the detection of PAstV in suspected animals in field areas. There are very less reports on the development of nucleo-diagnostic for PAstV but nucleo-diagnostics like real-time PCR and nPCR have been reported in the case of Human astroviruses (Dai *et al.* 2021). Identification of PAstV in Punjab is important for swine health and for economic returns as PAstV can lead to gastrointestinal diseases and poor growth rates, high mortality rates, and high veterinary expenses. So, nPCR can help in early detection of the virus and hence help farmers to reduce economic impacts incurred due to the spread of virus (Stäubli *et al.* 2021). In conclusion, nPCR is capable of giving a highly sensitive and specific identification of PAstV in swine populations that can help control the diseases, resulting in efficiency in production for farmers. Compared to other old methods, which are time-consuming and prone to contamination, nPCR assay displayed high sensitivity and specificity which is useful in conditions when there is low titer and high genetic variation of the virus.

ACKNOWLEDGMENT

The authors acknowledge the university authority for providing necessary facilities for the research work. The funding received under Rashtriya Krishi Vikas Yojana (RKVY) (Scheme: RKVY:13-4.1) is also duly acknowledged.

Table 3. Faecal samples from Pigs

S. No.	Diarrhoeic / Non-diarrhoeic	Conventional PCR (using outer set of primers)	Nested PCR results
P1	Diarrhoeic	Positive	Positive
P2	Diarrhoeic	Positive	Positive
P3	Diarrhoeic	Negative	Negative
P4	non-diarrhoeic	Positive	Positive
P5	non-diarrhoeic	Negative	Negative
P6	non-diarrhoeic	Positive	Positive
P7	non-diarrhoeic	Positive	Positive
P8	non-diarrhoeic	Negative	Positive
P9	non-diarrhoeic	Negative	Negative
P10	Diarrhoeic	Positive	Positive
P11	Diarrhoeic	Negative	Negative
P12	Diarrhoeic	Negative	Negative
P13	Diarrhoeic	Negative	Negative
P14	Diarrhoeic	Negative	Negative
P15	non-diarrhoeic	Negative	Negative
P16	non-diarrhoeic	Negative	Negative
P17	non-diarrhoeic	Negative	Negative
P18	Diarrhoeic	Negative	Negative
P19	non-diarrhoeic	Negative	Negative
P20	Diarrhoeic	Negative	Negative
P21	Diarrhoeic	Negative	Negative
P22	non-diarrhoeic	Negative	Negative
P23	Diarrhoeic	Positive	Positive
P24	Diarrhoeic	Negative	Positive
P25	Diarrhoeic	Negative	Negative
P26	Diarrhoeic	Negative	Negative
P27	Diarrhoeic	Positive	Positive
P28	Diarrhoeic	Positive	Positive
P29	Diarrhoeic	Negative	Negative
P30	Diarrhoeic	Negative	Negative
P31	Diarrhoeic	Negative	Negative
P32	Diarrhoeic	Negative	Negative
P33	Diarrhoeic	Positive	Positive
P34	Diarrhoeic	Negative	Negative
P35	Diarrhoeic	Negative	Negative
P36	Diarrhoeic	Negative	Negative
P37	Diarrhoeic	Negative	Negative
P38	Diarrhoeic	Negative	Negative
P39	Diarrhoeic	Negative	Negative
P40	Diarrhoeic	Negative	Negative
P41	Diarrhoeic	Negative	Negative
P42	Diarrhoeic	Negative	Negative
P43	Diarrhoeic	Negative	Negative
P44	Diarrhoeic	Negative	Negative
P45	Diarrhoeic	Negative	Negative
P46	Diarrhoeic	Negative	Negative
P47	Diarrhoeic	Negative	Negative
P48	Diarrhoeic	Negative	Negative
P49	Diarrhoeic	Negative	Negative
P50	Diarrhoeic	Negative	Negative

REFERENCES

Bae K S, Lee S, Lee J Y, Kim J H, Joo Y L, Lee S H, Chung H M and You K A. 2022. Development of diagnostic systems for wide range and highly sensitive detection of two waterborne hepatitis viruses from groundwater using the conventional reverse transcription nested PCR assay. *Journal of Virological Methods* **299**: 114344.

Caruana G, Croxatto A, Coste A. T, Opota O, Lamoth F, Jaton K and Greub G. 2020. Diagnostic strategies for SARS-CoV-2 infection and interpretation of microbiological results. *Clinical Microbiology and Infection* **26**(9): 1178–82.

Dai Y C, Xu Q H, Wu X B, Hu G F, Tang Y L, Li J D and Nie J. 2010. Development of real-time and nested RT-PCR to detect astrovirus and one-year survey of astrovirus in Jiangmen City, China. *Archives of Virology* **155**: 977–82.

Fang Q, Wang C, Liu H, Wu Q, Liang S, Cen M and Huang, W. 2019. Pathogenic characteristics of a porcine astrovirus strain isolated in China. *Viruses* **11**(12): 1156.

Foo P C, Nurul Najian A B, Muhamad N A, Ahamad M, Mohamed M, Yean Yean C and Lim B H. 2020. Loop-mediated isothermal amplification (LAMP) reaction as viable PCR substitute for diagnostic applications: a comparative analysis study of LAMP, conventional PCR, nested PCR (nPCR) and real-time PCR (qPCR) based on Entamoeba histolytica DNA derived from faecal sample. *BMC Biotechnology* **20**: 1–15.

Guix S, Bosch A and Pintó R M. 2013. Astrovirus taxonomy. *Astrovirus research: essential ideas, everyday impacts, future directions*. 97–118.

Hall TA. 1999. BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/ NT. *Nucleic Acids Symp Ser* **4**1: 95–98.

Jalal S, Hwang S Y, Kim C M, Kim D M, Yun N R, Seo J W and Lee S H. 2021. Comparison of RT-PCR, RT-nested PCRs, and real-time PCR for diagnosis of severe fever with thrombocytopenia syndrome: a prospective study. *Scientific Reports* **11**(1): 16764.

Kumthip K, Khamrin P, Saikruang W, Kongkaew A, Vachirachewin R, Ushijima H and Maneekarn N. 2018. Detection and genetic characterization of porcine astroviruses in piglets with and without diarrhea in Thailand. *Arch Virol*ogy **163**(7):1823–29.

Lambisia A W. 2021. Comparison of the Diagnostic Performance of TaqMan Array Cards, Enzyme Immunoassay, Real-Time PCR and Next Generation Sequencing in Investigation of Five Common Diarrhoea-Associated Enteric Viruses in Kilifi, Kenya (*Doctoral dissertation, JKUAT-COHES*).

Li C Q, Hu L Q, Liu G P, Wang Y, Li T, Chen S X and Zeng J G. 2023. A duplex nested RT-PCR method for monitoring porcine epidemic diarrhea virus and porcine delta-coronavirus. *BMC Veterinary Research* **19**(1): 151.

Meena H R and Singh Y P. 2013. Importance of information and communication technology tools among livestock farmers: A review. *Scientific Journal of Pure and Applied Sciences* **2**(2): 57–65.

Opriessni, T, Xiao C T and Halbur P G. 2020. Porcine astrovirus type 5-associated enteritis in pigs. *Journal of Comparative Pathology* **181**: 38–46.

Piryaei M, Bagheri S, Riahi A and Razmyar J. 2023. Astroviruses; Pathogenesis and Diagnosis: A Review. *Journal of Poultry Sciences and Avian Diseases* **1**(3): 1–17.

Puente H, Arguello H, Cortey M, Gómez-García M, Mencía-Ares O, Pérez-Perez L and Carvajal A. 2023. Detection and genetic characterization of enteric viruses in diarrhoea outbreaks from swine farms in Spain. *Porcine Health Management* **9**(1): 29.

Rawal G and Linhares D C. 2022. Scoping review on the epidemiology, diagnostics and clinical significance of porcine astroviruses. *Transboundary and Emerging Diseases* **69**(3): 974–985.

Salamunova S, Jackova A, Mandelik R, Novotny J, Vlasakova M and Vilcek S. 2018. Molecular detection of enteric viruses and the genetic characterization of porcine astroviruses and sapoviruses in domestic pigs from Slovakian farms. *BMC Veterinary Research* **14**: 1–9.

Staubli T, Rickli C I, Torgerson P R, Fraefel C and Lechmann J. 2021. Porcine teschovirus, sapelovirus, and enterovirus in Swiss pigs: multiplex RT-PCR investigation of viral frequencies and disease association. *Journal of Veterinary Diagnostic Investigation* **33**(5) 864–74.

Wildi N and Seuberlich T. 2021. Neurotropic astroviruses in animals. *Viruses* **13**(7): 1201.

Zhang Q, Wen D, Liu Q, Opriessnig T, Yu X and Jiang Y. 2023. Universal primer multiplex PCR assay for detection and genotyping of porcine astroviruses. *Journal of Virological Methods* **322**1: 114822.