Journal of Virological Methods 228 (2016) 103–107

Contents lists available at ScienceDirect

Journal of Virological Methods

journal homepage: www.elsevier.com/locate/jviromet

Rapid and sensitive detection of porcine torovirus by a reverse transcription loop-mediated isothermal amplification assay (RT-LAMP)

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Article history:
Received 7 July 2015
Received in revised form
15 November 2015
Accepted 16 November 2015
Available online 22 November 2015

Keywords:
Rapid detection
Porcine torovirus
Reverse transcription loop-mediated isothermal amplification assay

A B S T R A C T

Porcine torovirus (PToV) is associated with swine gastroenteritis, but its pathogenesis is uncertain because there is limited information regarding PToV due to its difficulty to adapt in vitro. This study has developed a rapid one-step reverse transcription loop-mediated isothermal amplification (RT-LAMP) method for the detection of PToV. A set of four primers specific to six regions within the PToV's highly conserved fragment of the M gene was designed for use with the RT-LAMP assay. The RT-LAMP assay was sensitive with a detection limit of 1×10^1 copies/μL, which was 100-fold higher than reverse-transcription PCR. No cross-reaction was observed with other similar viruses. A total of 175 clinical specimens were collected from the Sichuan province, and PToV was detected by the established RT-LAMP assay with a positive rate of 39.2% (69/175). This study developed the first rapid, sensitive, simple, cost-effective and accurate method for the detection of PToV. The results show that the RT-LAMP assay is highly feasible in clinical settings.

1. Introduction

Porcine torovirus (PToV) is a positive-sense, single-stranded polyadenylated RNA viruse belonging to the subfamily Torovirinae of the family Coronaviridae. There are four main torovirus species including equine torovirus (EToV), bovine torovirus (BToV), porcine torovirus (PToV) and human torovirus (HToV). Toroviruses, which infect humans, horses, cows, and pigs (Pignatelli et al., 2010b), have been detected in diarrheal fecal samples. In 1972, Torovirus was first isolated from a case of horse diarrhea samples in Switzerland and was named Berne virus (ETV) (Weiss, Steck, and Mc., 1983). In 1982, BToV was first isolated from a case of neonatal calf diarrhea in the United States. During this diarrhea epidemic approximately 15% of the infected cattle died (Woode et al., 1982). BToV had already been confirmed as one of the pathogens causing calf diarrhea in experimentally infected gnotobiotic calves and in field conditions.

In 1972, PToV particles were initially observed by electron microscopy from swine fecal samples in the Netherlands (Kroneman et al., 1998). After that, this pathogenic virus was investigated in many countries including Canada, South Africa, Italy, Belgium, and Hungary (Durham et al., 1989; Park et al., 2010; Pignatelli et al., 2010b; Scott et al., 1987; Vorster and Gerdes, 1993). Serological studies suggest a high prevalence of antibodies against PToV in Europe (Kroneman et al., 1998; Pignatelli et al., 2009; Weiss et al., 1983). Although previous reports indicate that toroviruses are dispersed widely throughout the globe and are highly epidemic in animals (MD, 2010; Park et al., 2010), there is little detailed information about them because it is difficult to adapt and produce toroviruses in vitro. The PToV genome organization is similar to other toroviruses. It is a positive-sense, single-stranded RNA molecule of approximately 25–30 kb, sharing 79% identity with Breda virus, organized into five ORFs expressing a replicase polyprotein (the 5′ two thirds end contains two large and overlapping open reading frames: ORFla and ORFlb) and four structural proteins: spike (S), membrane (M), hemagglutinin-esterase (HE)

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http://dx.doi.org/10.1016/j.jviromet.2015.11.009
0166-0934/

and nucleocapsid (N) (Aita et al., 2012; Kroneman et al., 1998; Pignatelli et al., 2009). Recently, a sequence data report for PToV strains showed that the N structural protein shared 90.6–93.1% amino acid sequence identity with Korean strains; the M structural protein was more conserved, sharing 98% amino acid sequence identity with the reported PToVs (Sun et al., 2014). In 2013, Lu et al. (2014) established a detection method on the basis of the M gene for PToV isolated from porcine fecal samples in Sichuan Province, China.

Loop-mediated isothermal amplification (LAMP) is an alternate amplification method that had been widely used to detect different infectious pathogens because of its rapid detection (Poon, 2005; Qiao et al., 2013; Zhang et al., 2013). Based on our work and that of others, we propose that the PToV RT-LAMP assay could target PToV gene M to build a new rapid, efficient and accurate molecular tool for detecting PToV virus in clinical specimens. We hope the RT-LAMP assay will help us better understand the prevalence of PToV in Sichuan province and the relationship between PToV and other diarrhea viruses.

2. Methods and materials

2.1. Viruses

PToV was obtained from the feces of piglets with diarrhea. All of these samples were collected from several different farms in Sichuan province. The following strains were provided by the Animal Biotechnology Center as controls: Porcine transmissible gastroenteritis virus (TGEV) T strain, porcine epidemic diarrhea virus (PEDV) CV777 strain, porcine pseudorabies virus (PRV) Fa strain, porcine rotavirus (RV) SC strain, porcine reproductive and respiratory syndrome virus (PRRSV) JXA1 strain, porcine kobuvirus (PKoV) SC2011 strain and Bovine torovirus (BToV) SC strain. All of these control strains are known to be related to PToV or cause similar clinical signs.

2.2. Design of the RT-LAMP and RT-PCR primers

Using the nucleic acid sequences of PToV published in GenBank (accession number: NC_022787.1), the sequence encoding the M gene was chosen as the target sequence for RT-LAMP and RT-PCR. Four primers specific for the M gene were designed with the Primer Explorer V4 software using default settings (http://primerexplorer.jp/elamp4.0.0/index.html). They included an outer pair (F3, B3) and an inner pair (FIP, BIP) of primers. A pair of primers (named P1 and P2) was also used for the RT-PCR amplification of the M gene that was designed with Primer 5 software. Information regarding the primer names and sequences is shown in Table 1.

2.3. Extraction of nucleic acids

Fecal samples were transported to the laboratory within 1 h and stored on ice at −70 °C. Prior to RNA extraction, approximately 0.1 g of feces was dissolved using a homogenizer in 1.9 ml of 0.9% saline water. These samples were clarified by low-speed centrifugation at 4000 × *g* for 20 min. RNA was extracted from 400 μL of the feces suspension liquid for clarification using TRIzoI LS or TRIzoI reagent (Invitrogen, USA) according to the manufacturer's instructions. The resulting RNA particles were dissolved in 20 μL diethylpyrocarbonate (DEPC)-treated water. Single stranded cDNA was synthesized by reverse transcriptase (RT) using the TaKaRa Reverse Transcription System (TaKaRa Dalian Biotechnology, Dalian, China). Total RNA and genomic DNA were quantified using standard methods to measure the OD value. Both the RNA and cDNA were stored at −20 °C before use.

2.4. Optimization of RT-LAMP conditions

The RT-LAMP assay was performed using the Loopamp RNA Amplification Kit (RT-LAMP) under the following conditions: 1 μL sample (RNA or DNA), 0.2 μM each of F3 and B3, 1.6 μM each of FIP and BIP, betaine 3 μL (8.0 mmol/L, Sigma–Aldrich, St. Louis, MO, USA), 1.4 mM of dNTPs, 1 μL of Bst DNA polymerase (8 U/μL, New England Biolabs, MA, USA), 0.75 μL of M-MuLV reverse transcriptase (200 U/μL, Promega, USA), 0.5 μL of RNasin (40 U/μL, Promega, USA), 12.5 μL of LAMP buffer (20 mM Tris–HCl [pH 8.8], 8.0 mM $MgSO_4$, 10 mM KCl, 10 mM $(NH_4)_2S0_4$, 0.1%Triton X-100), and DEPC-treated water was added to obtain a final volume of 25 μL. DEPC-treated water was also set up as a negative control. The initial amplification reaction was performed in a PCR reaction tube using a heating block incubated at 65 °C for 60 min, followed by 10 min at 80 °C to terminate the reaction. 5 μL of amplification product was used to run 20 g/L agarose gel electrophoresis at 110 V for 20 min followed by imaging analysis using a UV gel imaging system. Electrophoresis of RT-LAMP products showed characteristic ladder shaped banding.

2.5. RT-PCR

The PCR was carried out using 25 μL of a PCR mix containing 12.5 μL of 2× Taq PCR Master Mix (Biomed, Beijing, PR China), 2 μL each of forward and reverse primers (10 μmol/L), 5 μL of cDNA and DEPC-treated water. The reaction program was 94 °C for 5 min; 94 °C for 30 s, 57 °C for 30 s, 72 °C for 30 s, 35 cycles and a final extension cycle at 72 °C for 10 min. The products were analyzed using agarose gel electrophoresis (1% agarose, Tris–acetate–EDTA (TAE) buffer) and stained with GoldView.

2.6. Specificity and sensitivity of RT-LAMP assay

To synthesize the control RNA for RT-LAMP, the nucleocapsid protein sequence of the PToV M gene was amplified and the RT-PCR products were recovered, purified, and cloned into E.coli DH5α using the pGEM-T Easy Vector (Promega, Fitchburg, WI, USA). Then, the linearization of cDNA by primers P1 and P2 allowed for transcription in vitro, and the amplicons were gel-purified and used as templates for transcription with T7 RNA polymerase (TaKaRa, Dalian, PR China) according to the manufacturer's instructions. Using TaKaRa MiniBEST Plasmid Purification Kit Ver.4.0 for extraction of the plasmid, the PToV positive plasmid's

Table 1
The RT-LAMP primers for PToV detection.

Method	Primer No.	Primer sequence	Length	Genome position[a]
	F3	GCAATTTTAGTTAAAGAAGT	20	460–480
	B3	CAACTGCGTATAATCAGCG	29	663–681
RT-LAMP	FiP	GTCCACTAACACAAATCTTAAGTGC-AGAGTATTGTAGCAAACCATTGA	48	501–523
	BiP	TACTCGGTCATCAACCACAAGT-CCCTGCAAAATAAAAAGATGG	43	639–659
RT-PCR	P1	TCGTGGTCCAATTGTTTTAGTAG	23	399-421
	P2	CTACTCAAACTTAACACTTGA	21	682–702

[a] Genome position refers to porcine torovirus: GenBank KF727584.1.

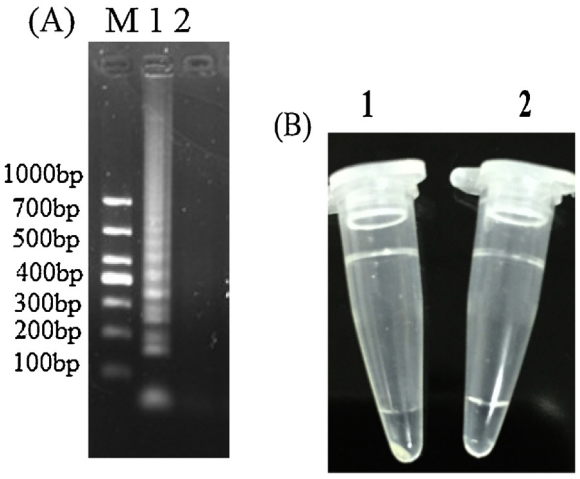

Fig. 1. (A) Agarose gel electrophoresis, M: DL 1000 DNA Marker, Lane 1: pMD19-M; Lane 2: Negative control. (B) Magnesium pyrophosphate precipitation detection, Lane 1: pMD19-M; Lane 2: negative control.

concentration was determined by micro-spectrophotometer analysis and stored at −70 °C. To determine the sensitivity of RT-LAMP assay, the PToV positive plasmid template was diluted 10-fold (from 1×10^0–1×10^8 copies/μL) and amplified by RT-LAMP and RT-PCR assays. The RT-LAMP amplification products were detected by 20 g/L agarose gel electrophoresis and visualized under UV light or visually inspected by adding SYBR Green I (Invitrogen, Madison, USA). The RT-PCR amplification products were detected by 10 g/L agarose gel electrophoresis.

To determine the specificity of the RT-LAMP assay, viruses related to PToV or known to cause similar clinical symptoms, including TGEV, PEDV, PRV, RV, PRRSV, PKoV and BToV were tested together with PToV. Distilled water was also used as a negative control. The products were detected by 20 g/L agarose gel electrophoresis or were observed visually by adding SYBR Green I (Invitrogen, Madison, USA).

2.7. Preliminary testing of clinical samples

A total of 175 fecal samples were collected from swine diarrhea in different regions of Sichuan Province (including Chengdu, Mianyang, Yaan, Ziyang, Meishan, Suining, and Deyang), during the winter from 2013 to 2014. Most of the sampled piglets were 1–3 weeks old, although there were also samples from 3 to 11 week old piglets and from piglets that were more than 11 weeks. Approximately 30 samples came from healthy piglets. The RT-LAMP method and conventional RT-PCR assays were used for testing.

3. Results

3.1. PToV RT-LAMP and RT-PCR

After using the PToV recombinant plasmid as a template to perform the PToV RT-LAMP assay with the reaction components and visualizing RT-LAMP reaction products through agarose gel electrophoresis analysis, the results showed that the amplicon bands manifested to approximately the size of 159 bp,. However, the negative control bands disappeared after amplification (Fig. 1A). The positive reaction tube appeared to have white precipitate at the bottom after the instantaneous centrifugation, but the negative control tube did not have any precipitate or coloring after centrifugation (Fig. 1B).

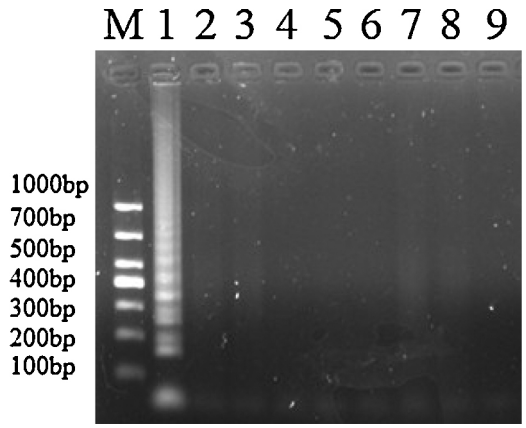

Fig. 2. LAMP products detected by agarose gel electrophoresis were strained by GoldView. Lane M: DNA marker DL1000; Lane 1: PToV; Lanes 2–8: TGEV, PEDV, PRV, RV, PRRSV, PKoV, BToV, respectively; Lane 9: negative control.

3.2. Specificity of the PToV RT-LAMP

This type of RT-LAMP method was applied to PToV, TGEV, PEDV, PRV, RV, PRRSV, and PKoV viruses for nucleic acid amplification to test the specificity of the RT-LAMP detection method. To assay the specificity within torovirus species, samples containing BToV were also tested (Pignatelli et al., 2009). The resulting products were visualized on an agarose gel following electrophoresis. Only the RT-LAMP of the PToV recombinant plasmid template resulted in amplification as evidenced by a ladder-like pattern on the gel. In contrast, no RT-LAMP products were detected from the 7 other viruses tested (Fig. 2). Data mentioned above showed that the RT-LAMP method was established with high specificity.

3.3. Sensitivity of the PToV RT-LAMP

To compare the correlation of the sensitivity of RT-LAMP and RT-PCR, a dilution series of PToV recombinant plasmids was used as a template to conduct the RT-LAMP assay and RT-PCR. The RT-LAMP and RT-PCR amplified products were analyzed by agarose gel electrophoresis (2% agarose, TAE or 1% agarose, TAE), and stained with GoldView. If dye SYBR Green I is added to the LAMP reaction solution, the result can be observed visually (Notomi et al., 2000). The color of the RT-LAMP reaction containing PToV changed from orange to green, while the RT-LAMP reaction solution without PToV had no color change (Fig. 3A). Conventional RT-PCR was used to detect the sensitivity limit of 1×10^3 copies/μL (Fig. 3C) and the detection sensitivity of RT-LAMP method for 1×10^1 copies/μL (Fig. 3B). The results showed that the detection sensitivity of RT-LAMP method is 100 times that of the conventional RT-PCR detection method.

3.4. Evaluation of RT-LAMP assays on clinical samples

To assess the evaluation of the RT-LAMP assay for detecting PToV in clinical material, approximately 175 fecal samples were collected (30 samples came from apparently healthy piglets and approximately 145 fecal samples were collected from piglets with diarrhea). These samples were tested using both the RT-LAMP assay and as well as conventional RT-PCR. The results of these two methods are summarized in Table 2. There was no false positive from the two methods among the 175 samples. For the 175 suspicious samples, the RT-LAMP method gave a total of 69 positive results. The positive rate for the entire sample set was 39.2% (69/175) with 65 out of 145 diarrhea samples and 4 out of 30 apparently healthy samples testing positive for PToV (Table 2). On the other hand,

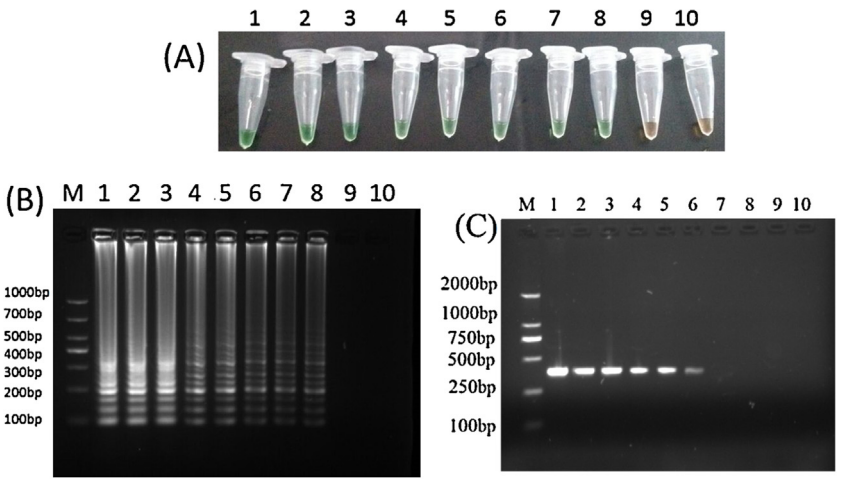

Fig. 3. Comparing the sensitivity of the PToV-RT-LAMP and PToV-RT-PCR assays. (A) PToV-RT-LAMP products detected by 1000× SYBR Green I. (B) PToV-RT-LAMP products detected by agarose gel electrophoresis strained by GoldView. Lane M, DNA marker DL1000. Lanes 1–9: RNA of PToV with 10-fold dilutions (1×10^8–1×10^0 copies), and Lane 10: negative control. (C) PToV-RT-PCR products detected by agarose gel electrophoresis strained by GoldView. Lane M: DNA marker DL2000; Lanes 1–9: the RNA of PToV with 10-fold dilutions (1×10^8–1×10^0 copies), and Lane 10: negative control.

Table 2
The PToV detection results in Sichuan province based on PToV-RT-LAMP and PToV-RT-PCR.

Origin of samples	Samples	Number of samples	Detection results	
			RT-LAMP (positive/total sample)	RT-PCR (positive/total sample)
Chengdu	Diarrhea feces	25	8 (32.0%)	6 (24.0%)
	Healthy	5	1 (20.0%)	1 (20.0%)
Mianyang	Diarrhea feces	30	17 (56.7%)	14 (46.7%)
	Healthy	4	1 (25.0%)	0
Ya'an	Diarrhea feces	15	6 (40.0%)	4 (26.7%)
	Healthy	3	0	0
Ziyang	Diarrhea feces	10	4 (40.0%)	3 (30.0%)
	Healthy	4	0	0
Suining	Diarrhea feces	20	9 (45.0%)	6 (20.0%)
	Healthy	5	1 (20.0%)	1 (20.0%)
Meishan	Diarrhea feces	25	13 (52.0%)	9 (36.0%)
	Healthy	5	1 (20.0%)	0
Deyang	Diarrhea feces	20	8 (40.0%)	6 (30.0%)
	Healthy	4	0	0
Total	Diarrhea feces	145	65 (44.9%)	48 (33.1%)
	Healthy	30	4 (13.0%)	2 (6.7%)

the positive rate for the entire sample set was 28.6% (50/175) by conventional RT-PCR. 48 out of 145 diarrhea samples and 2 out of 30 apparently healthy samples tested positive for PToV (Table 2). These results indicated that RT-LAMP assay exhibited better sensitivities than RT-PCR when detecting PToV in field samples.

4. Discussion

PToV, an unfamiliar small RNA virus, has been discovered in feces from piglets with diarrhea in recent years. Since the winter of 2010, there were epidemic outbreaks in piglets with watery diarrhea and vomiting as the main clinical symptoms. This caused high morbidity and mortality in most parts of China, which in turn caused great economic losses in the pig industry (Li et al., 2014; Lu et al., 2014). Most of the sampled piglets of 1–3 weeks of age were more vulnerable to PToV than the others (Zhou et al., 2013), and antibiotic treatment was invalid in all sampled piglets. Through inspection, there were many similarities with PEDV infections. In China at the end of 2010, researchers were trying to study the etiology of the diarrhea in piglets by comparing relevant pathogen outbreaks such as PEDV, TGEV, RV, and PKoV to find a correlation between them (Li et al., 2014; Wang et al., 2012). To determine if PToV played an important role in this epidemic situation, it is important to set up a sensitive and rapid method to detect PToV.

To date, PToV currently has no suitable model in vitro, so PToV studies only were focused on epidemiological investigation. Researchers established some diagnostic methods for PToV, such as conventional RT-PCR, nested PCR, real-time PCR and indirect ELISA (Alonso-Padilla et al., 2012; MD, 2010). However, conventional RT-PCR requires not only a certain concentration of RNA but also 2–2.5 h and special equipment to complete. The RT-LAMP is considerably more time-efficient because the optimal time needed is 1 h. Moreover, unlike other methods, the RT-LAMP assay is carried out in a heat block or constant temperature water bath, and does not need a thermocycler. Therefore, it is more affordable for clinical testing.

A critical factor when performing the RT-LAMP assay is to select a conserved nucleic acid fragment for the design of specific primers (two outer primers F3 and B3 and two inner primers FIP and BIP). The technology relies on the six primers, which allow for specific areas in the target sequence to be identified. Then the BstDNA polymerase is required to complete the nucleic acid amplification reaction, which has the function of unwinding. Amplification of target sequences could be efficient (which can be achieved as much as

10^9–10^{10} copies in a short time), rapid (30–60 min), and specific at a constant temperature (60–65 °C) (Wang et al., 2012). Agarose gel electrophoresis and real-time monitoring using a turbidimeter can accomplish the detection of positive amplification of RT-LAMP. In addition, amplification of the target gene can also be visualized by adding SYBR Green I to the reaction mixture.

This study compared the sensitivity of the RT-LAMP assay with RT-PCR for detecting the PToV. The RT-LAMP assay was able to detect PToV at a dilution of 1×10^1 copies while the conventional RT-PCR was able to detect PToV at 1×10^3 copies indicating that the sensitivity of RT-LAMP was 100-fold higher than RT-PCR. This study also compared the specificity of the RT-LAMP assay. Specific test results show that the RT-LAMP assay had a positive result only in the PToV recombinant plasmid and there was no amplification obtained from other viruses. This showed that the RT-LAMP assay had strong sensitivity and very high specificity.

A recent report indicated that PToV infection had no clinical signs and had a high infection rate in China. Of 175 clinical specimens from Sichuan province, 39.2% (69/175) of the samples tested positive for PToVs by RT-LAMP, while conventional RT-PCR only identified 50 PToV positive samples. This shows that the RT-LAMP assay is more sensitive and accurate. Mianyang and Meishan have the highest detection rate of PToV of fecal diarrhea in pigs testing positive for PToV with 56.7% and 52.0%, respectively. Chengdu has the lowest detection rate with only 32.0% of pigs positive for PToV. There are approximately 5 out of 30 samples collected from healthy piglets that tested positive for PToV by RT-LAMP assay, but the using conventional RT-PCR only 2 samples tested positive. This is a large difference from the earlier reports on the detection of PToV by conventional RT-PCR. Early reports in China showed the detection level at 37.96% using RT-PCR (331/872) (Lu et al., 2014). In Korea it was 6.4% (19/295) (Shin et al., 2010), and on a Spanish farm it was 39.6% (19/48) (Pignatelli et al., 2010b). One explanation for this is that samples collected from pigs with lower amounts of PToV may have had too little virus to be detected by the less sensitive RT-PCR method. Another reason may be that PToV are not apparent in subclinical infections and can survive in feces for a short time.

Torovirus is widely detected as a pathogen responsible for the intestinal diarrhea disease in animals and human beings (Maestre, Garzón, and Rodríguez, 2011; Oberste et al., 1999). In recent years, the epidemiological data about PToV has also been released in many countries (Pignatelli et al., 2010a; Shin et al., 2010) indicating that it may be widely circulating around the world and with high prevalence. Most PToV infections seem to be subclinical. At present, it is difficult to grow in cultured cells, so a fast, simple, sensitive detection method is extremely urgent.

5. Conclusion

Most PToV infections in animals appear to be subclinical, and the relationship between PToV infections with other enteric pathogen causing diarrhea remains unclear. However, the virus is still a potential threat for humans. Our newly developed molecular diagnostic method based on RT-LAMP could be more suited for sensitive and rapid detection of PToV and very useful in performing more extensive epidemiological studies that could provide the basis for prevention of PToV in the future.

Ethical approval

All sampling procedures were reviewed and approved by the Institute of Animal Health Animal Care and Use Committee at Sichuan Agricultural University.

Acknowledgements

This study was supported by Sichuan Youth Waterfowl Disease Control and Prevention Science and Technology Innovative Research Team (Project No: 2013TD0015) and Changjiang Scholars and Innovative Research Team in University (Project No: IRT13083), program for New Century Excellent Talents of Ministry of Education of China (NCET 11-1059).

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