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RT-LAMP Test Kit: A New Generation of Molecular Quick Test Kit for Porcine Epidemic Diarrhea Virus (PEDV)

Clarissa Yvonne J. Domingo and Rubigilda Paraguison-Alili

College of Veterinary Medicine, Central Luzon State University Science City of Muñoz, Nueva Ecija

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The current invention is an RT-LAMP test kit that is exceptionally simplified and economically designed for detecting porcine epidemic diarrhea virus (PEDV), a number one threat causing an infectious acute enteritis and diarrhea in pigs, severely in piglets. The test is based on the principle of Loop- mediated Isothermal Amplification method (LAMP) which is recognized by the World Assembly of Delegates of the OIE in May 2012 as an alternative technique to PCR. The RT-LAMP test kit is a closed- tube system designed with built-in less than a minute procedure nucleic acid extraction procedure devoid of using commercial kit done in less than a minute and the economically formulated components of the RT-LAMP premixes which are both assembled in one kit. The kit specifically contains originally designed oligonucleotides, locally engineered plasmid DNA as positive control, LAMP reagent premix and the simplest nucleic acid extraction process.
The study designed new LAMP primers and optimized the reverse transcription LAMP protocol; validated the RT-LAMP protocol using field samples for surveillance data of PED; determined the prevalence of PED infections among hog farms in two provinces of the country using the developed RT- LAMP assay; established the sensitivity and specificity of the developed RT-LAMP protocol with RT- PCR; constructed DNA plasmid harboring the target DNA region of the S gene of the virus for reference template or positive control to be incorporated in the kit; and finally, had the PED LAMP test kits validated by other diagnostic laboratories.
The optimized PED RT-LAMP protocol could amplify at 65oC for 30 minutes using a heat block.
The analytical sensitivity of RT-LAMP which can detect the lowest PED virus concentration was at 0.00031 ng/µl concentration or 10-6 dilution. The outer forward and reverse primers of RT-LAMP used in qPCR are specific for PED virus amplification through the unified peaks at melting temperature above 80.0oC. Analytical specificity showed that RT-LAMP was specific for the spike glycoprotein target gene of PED virus only and could not amplify other swine gastrointestinal genes. Using the cloned cDNA as reference template in RT-PCR, a PCR product with an amplicon size of 188 bp was visualized in the gel and the DNA sequence found it to be 99% homologous to the target gene. PED prevalence in the two provinces using the RT-LAMP assay was 65.3% (95% CI: 60.0-70.5). Diagnostic sensitivity, with RT- PCR as the gold standard, RT-LAMP was 100% whereas specificity was 63.64 up to 75.0%. This is because the RT-PCR assay could not detect the virus in samples that were positive in the RT-LAMP assay. The RT-LAMP premix can still efficiently amplify even up to 80 days of cold storage (-20oC). Field validation of the RT-LAMP test kit was conducted and PED prevalence was 62% using fecal samples. The test kit was also used for intestines of infected piglets, swabbing from empty feed sacks, soiled floors and farrowing crates. Two evaluators who came from independent animal diagnostic laboratories indicated their satisfaction towards the LAMP

after they were allowed to conduct the RT- LAMP assays themselves.

Introduction

Porcine epidemic diarrhea (PED) is an infectious enteric disease characterized by acute enteritis and diarrhea in pigs, the infection is more severe in piglets. The causative agent of PED is porcine epidemic diarrhea virus (PEDV), an enveloped and single-stranded RNA virus that belongs to the family Coronaviridae.

In the Philippines, in May to August 2010 an outbreak of Porcine Epidemic Diarrhea (PED) occurred in the province of Batangas killing 67% (11,414 head) of 17,115 sick pigs. The economic losses due to this outbreak amounted to PhP 9.131M. Currently, the cost of diagnostic test for PED using PCR (the gold standard) is PhP 3,000.00/sample. With this cost, surveillance would be impractical and uneconomical, with this condition, there is a need for a simple, rapid and economical diagnostic test to detect the virus even prior to appearance of symptoms so that early detection can help the hog raisers apply preventive measures to stop the spread of the disease and reduce the total number of loss during a farm outbreak. Assuming that 50% of the 11,414 pigs affected by PED in Batangas survived due to early detection, economic losses can be reduced by P4.56M.At present, and several methods are used to detect these harmful microorganisms. Among these, PCR (polymerase chain reaction) is the most effective and sensitive. This test enables specific identification of the microorganism directly from body fluids or manure. However, it is costly at P3,000.00/sample, time-consuming and labor intensive.

The LAMP-based test can be accomplished in simple laboratories with limited resources and by anyone with basic laboratory skills. It is more sensitive than PCR because it can detect the DNA from 1 cell in the sample as compared to PCR which requires 100,000 cells before a positive result can be observed (Notomi et al., 2000). The cost per test is around P250.00 per sample hence, cheaper than current tests.

The test can detect the presence of a particular pathogen even before symptoms appear thus, appropriate preventive measures can be instituted and it will improve pre-weaning and post weaning survival by up to 90%, thus increasing pork production and preventing unscrupulous trading of hot meat.

Normally, the amount of reagents to run a LAMP test is 25 microliter (µl). In this study, this amount was reduced to 12.5 µl or 6.25 µl. Moreover, all LAMP reagents are now combined in one vial ready to be mixed with the sample. In addition, LAMP primers were designed to detect the virus that cause diarrhea. LAMP reaction is carried out in one temperature for a given period of time using a water bath or a heat block that remains a permanent fixture in the laboratory. In the conventional reading of LAMP results, they

can be visualized by the naked eye based on color change (from orange to green) after the addition of a dye, by the presence of fluorescence (glowing greenish-white light) after observing with a handy UV light viewer or one may opt to confirm the results by analyzing the presence of multiple ladder-like bands using gel electrophoresis. LAMP-based tests, as evidenced by the results of this study, is a new platform for detecting the PED virus or it may be applied in diagnosis of any other pathogens there is.

Review of Literature

Two critical disease problems of the local swine industry are respiratory and gastrointestinal infections of pigs of all ages. It could be due to viral, bacterial and parasitic pathogens, or a combination of two or all of these pathogens. For quite some time now, these diseases have been causing significant reduction in swine farm productivity and efficiency, and income. Affected growing herds can lead to delays of 14 - 21 days in pigs reaching finishing weight. They suffer from reduced growth rate, low feed efficiency and high mortality.

According to Yang *et al.* (2009), the key for controlling respiratory and gastrointestinal diseases is to obtain a correct diagnosis of the causative agent. Diagnosis can be made by identification of pathological lesions, isolation from clinical samples, serotyping, antibody detection, molecular biological methods and immuno-histochemical diagnosis.

Isolation of the pathogen from clinical samples faces the possibility of no growth especially if antibiotics have been given to sick animals, except if resistance against such antibiotics has built up over time. Likewise, bacterial culture and isolation is time consuming, expensive and results can only be seen after several days of confirmatory testing for identification.

Since pathogen isolation from clinical samples is difficult due to contamination by less demanding microorganisms, immuno-histochemical (IHC) examination is recommended for diagnosis of respiratory and gastrointestinal infections. IHC allows detection of non-living microorganisms in the cytoplasm of phagocytes. However, some polyclonal antibodies used for diagnosis of the pathogen in question by the IHC method may cross react with other related microorganisms (Nedbalcova *et al.*, 2010).

Due to the fact that it is not always possible to obtain a pure culture of the pathogen in question, a molecular biological method such as PCR, was developed that enables specific identification (102 CFU/ml) of the microorganism directly from clinical samples, and moreover, it can detect non-living organisms.

Currently, a molecular technique called loop-mediated isothermal amplification (LAMP) of DNA has evolved to diagnose all kinds of pathogen (virus, bacteria, fungi, yeasts, protozoa, helminths). This was first

developed by Notomi *et al.* (2000). The technique uses four to six primers that recognize six to eight regions of the target DNA, respectively, in conjunction with the enzyme Bst polymerase, which has strand displacement activity. The simultaneous initiation of DNA synthesis by multiple primers makes the technique highly specific. The LAMP test is carried out under isothermal conditions (60–65oC) and produces large amount of DNA (Notomi *et al*., 2000). The reaction shows high tolerance to biological products (Kaneko *et al*., 2007), meaning that DNA extraction may not be necessary (Poon *et al*., 2006) and the product can be inspected visually by the addition of SYBR Green I (Iwamoto *et al*., 2003; Yoshida *et al*., 2005).

The causative agent of PED is porcine epidemic diarrhea virus (PEDV), an enveloped and single-stranded RNA virus that belongs to the family Coronaviridae. Coronavirus comprises three major viral structural proteins: Nucleocapsid (N) protein, Membrane (M) protein, Spike (S) protein. The S protein is a major viral antigen, binds to a cellular receptor for virus attachment to enter target cells and mediates viral attachment to target cells which served as the source of the target gene for the designed oligonucleotides. Briefly, LAMP proceeds when the forward inner primer (FIP) anneals to the complementary region (F2c) in the target DNA and initiates the first strand synthesis, and then the outer forward primer (F3) hybridizes and displaces the first strand, forming a loop structure at one end (Notomi *et al*., 2000). This single- stranded DNA serves as template for backward inner primer (BIP)-initiated DNA synthesis and subsequent outer backward (B3)-primed strand displacement DNA synthesis, leading to the formation of dumbbell-shaped DNA structures (Notomi *et al*., 2000). The stem-loop thus formed acts as a template, and subsequently one inner primer hybridises to the loop on the product and initiates the displacement DNA synthesis, forming the original stem loop and a new stem loop that is twice as long (Yamada *et al*., 2006). The final products are stem-loop DNAs with several inverted repeats of the target DNA, and cauliflower-like structures bearing multiple loops (Notomi *et al*., 2000).

Characteristics of LAMP:

1. There is no need for a step to denature double stranded into a single stranded form.
2. The whole amplification reaction takes place continuously under isothermal conditions.
3. The amplification efficiency is extremely high.
4. By designing 4 primers to recognize 6 distinct regions, the LAMP method is able to specifically amplify the target gene.
5. The total cost can be reduced, as LAMP does not require special reagents or sophisticated equipment.

6. The amplified products have a structure consisting of alternately inverted repeats of the target sequence on the same strand.
7. Amplification can be done with RNA templates following the same procedure as with DNA templates, simply through the addition of reverse transcriptase.

Detection methods for LAMP products

Magnesium pyrophosphate, a by-product of the amplification process, is produced in proportional amounts to the amplified product. LAMP is highly efficient and large amounts of end products are synthesized, altogether with large amounts of pyrophosphate. The white turbidity attributed to it may be visually observed. Therefore, the presence of turbidity indicates the formation of the targeted genomic region. Visual detection can also be achieved by adding an intercalating dye, most often SYBR Green I, to the final reaction mixture. When UV light is applied the presence of specific product would be recognized by green fluorescence. A real-time follow up of the reaction can be achieved as turbidity can be measured with an instrument called turbidimeter. This allows for quantitative detection of pathogens. If an agarose gel electrophoresis of the LAMP product is performed, bands with various sizes will be visualized at regular intervals. Additionally, restriction analysis alone or followed by sequencing reactionscan be performed for verification of new protocols that are tested for the first time (Parida *et al*., 2008).

Advantages of LAMP

Under optimal conditions the LAMP reaction produces a tremendous amount of a targeted sequence (109-1010 copies) for less than an hour (Notomi *et al*., 2000). The process is isothermal and can be carried out in a simple thermostat or water bath. The method is comparable to PCR in terms of sensitivity, but is less affected by presence of non-targeted DNA and inhibitory molecules. Some researchers even report specific amplification with LAMP without prior extraction procedure, by directly adding reaction mixture to swab specimens or sera (Ihira *et al*., 2007; Yamada *et al*., 2006). LAMP is highly specific as six separate genomic regions in the initial stage and four in the later steps, need to be recognized in order that the reaction is carried out. Therefore, a successful reaction would indicate the presence of the targeted sequence.

Tolerance of LAMP to inhibitory substances

Hemoglobin, heparin, EDTA, and IgG have been previously reported as inhibitors for Taq DNA polymerase used in PCR, whilst Bst DNA

polymerase used in LAMP has been presumed to be unaffected by these inhibitors based on results obtained on blood blotted filter papers. Boehme *et al*. (2007) have also reported that LAMP reactions were not inhibited when sputum samples with blood were used as templates when LAMP was used in clinical diagnosis for pulmonary diagnosis. It is further confirmed that LAMP has superior tolerance to these inhibitors as compared to PCR, whereby PCR was inhibited by minimal concentrations of haemoglobin, IgG and IgM, whilst none inhibited LAMP. Additionally, Kaneko *et al*. (2007) have reported that LAMP has superior tolerance than PCR to substances used in in vitro cultures including saline, phosphate buffered saline (PBS), Eagle's minimum essential medium (MEM), aqueous and vitreous humour, and that it is only inhibited by more than 1% of each serum, plasma and urine.

Comparison of LAMP with other high end diagnostic tests

Table 1 shows how the LAMP based assay compares with other high end diagnostic tests.

Parameters	RT-LAMP	RT-PCR	ELISA
Sensitivity (%)	98 (detects DNA from 1 cell in the sample)	94 (detects DNA from 100,000 cells)	>90 (detects antibodies not antigens)
Specificity (%)	99	97	>90
Time required (hr)	45 mins	4 hours	4 hours
Equipment needed	Heat block	Thermocycler, gel electrophoresis, gel documentation	Incubator, ELISA reader & washer
Sensitivity to inhibitors or reliability	Bst polymerase is not affected by contaminants	Taq polymerase affected by inhibitors, liable to give false results	Reliability is affected by inhibitors or contaminants
Cost of equipment (P)	18,000 to 30,000	500,000	800,000
Cost/sample or reaction (P)	250.00	3,000.00	350.00

The general objective of this research study is to develop a diagnostic kit for swine viral enteric infection particularly the Porcine Epidemic Diarrhea Virus (PED-V) using the Reverse Transcription Loop-mediated Isothermal Amplification method (RT-LAMP) and assemble into a convenient test kit - the Andali RT-LAMP Test kit for PED. Specifically, this study attempted to:

- Design new LAMP primers and optimize LAMP protocols for PED infections of swine;
- Validate developed RT-LAMP protocol using field samples for surveillance data of PED;
- Determine prevalence of PED infections among hog farms in regions 3 and 4 using the developed RT-LAMP assay;
- Establish sensitivity and specificity of the developed RT-LAMP protocol with RT-PCR;

- Construct DNA plasmid harboring the target DNA region of the S gene of the virus for reference template or positive control to be incorporated in the kit; and finally,
- Validate of the PED LAMP test kits by other diagnostic laboratories.

Methodology

Primer Design

Porcine epidemic diarrhea virus (PEDV) is an enveloped and single-stranded RNA virus that belongs to the family Coronaviridae. Coronavirus comprises three major viral structural proteins: Nucleocapsid (N) protein, Membrane (M) protein, Spike (S) protein. The S protein is a major viral antigen, binds to a cellular receptor for virus attachment to enter target cells and mediates viral attachment to target cells which served as the source of the target gene for the designed oligonucleotides. Following the NCBI website http:.//www.ncbi.nlm.nih.gov/, search PEDV published in Genbank. Using BLAST PEDV nucleotide sequence was selected from the Genbank accession number KM 406181.1. Using the LAMP Primer Explorer®web-interface available at http://primerexplorer.jp/e/ LAMP primers were designed, selected from the generated sets of primers. From the selected set, each primer was analyzed using the NCBI BLAST to check the specificity and sequence availability in other organisms, microorganisms and viruses.

Technical Aspect of the PED RT-LAMP Kit

Nucleic Acid Isolation which is technically incorporated in the kit is performed by crushing the clinical sample, preferably fecal sample of about 10µ L or a toothpick tip dipped. In a 1.5-mL microcentrifuge tube, using a clean and sterile mini grinder, the sample is crushed and extracted with alkaline method and no commercial kit was needed. An RT-LAMP assay method for detecting porcine epidemic diarrhea virus (PEDV) was achieved with the following components: The 6.5 to 12.5-µL RT-LAMP reaction reagent composition all combined in single reaction tube and just 1 µ L RNA extract is added wherein the concentration of RT-LAMP reaction solution comprises of: LAMP buffer, dNTP mix, Bst DNA polymerase and a reverse transcriptase. Primer mix is composed of inner primers F3 and B3 primers, outer primers FIP and BIP and the loop primers F-loop and B-loop. For each formulated RT-LAMP reagent mix, 1 µL of nucleic acid extract is added. It is then incubated at 63°C to 65°C for 30 minutes into a heat block, water bath or any improvised heating instrument which can be set with the recommended temperature that can be utilized in field deployment. After completion of the reaction, SYBR Green I fluorescent dye, which is also

included in the kit, is added and change in color reaction is observed. Green indicates the presence of the PEDV and if it remains orange in color, it indicates negative reaction.
Locally engineered positive control, construction of plasmid incorporating the target DNA region harboring the corresponding target PCR product generated using the F3 and B3 of the LAMP primer set. Transformation and DNA cloning were performed to produce clones of the DNA region from the PED virus S gene which served as positive control in every assay. The PCR products were ligated to the PGEM□T-easy plasmid vector of the Promega according to the protocol suggested by the manufacturer. Transformation was performed using the JM109 competent cells of Promega. The resulting plasmid construct clones were finally confirmed for DNA sequencing prior to application in every LAMP assay.

Validation of quick test kits

During the development of the kits, several formulations are tested.
1. Validation in the research laboratory
The key factors to the selection and validation of the molecular tests are diagnostic utility, analytical performance in service and economic factors.

Validation and sensitivity protocol will include the following factors:
a. These kits were compared with RT-PCR and qPCR method
b. Variability in storage temperatures are tested.
c. The number of replicates for the tests were analyzed to determine repeatability and the range of the conditions of the mixtures.

2. Validation in other animal diagnostic laboratories
After an exhaustive test in the research laboratory (at least three different laboratories were given an opportunity to use the kits and required to submit their validation reports).

Validation Results Report:
Upon completion of the process a validation report were generated given the limitations documented from the research lab tests.

Survey swine enteric infections in regions 3 and 4 using the quick test kits

Establishment of linkages with swine farms in Regions III and IV. The selection of commercial and small swine farms in the two regions were made in coordination with the provincial/municipal veterinarians. Report on cases of gastrointestinal infections with symptoms of diarrhea were obtained from the veterinary offices. A list of affected farms were obtained and used in constructing the

sampling frame.

Sensitivity and Specificity Comparison of RT-LAMP with RT-PCR and qPCR

The specificity of PED-LAMP were examined by the use of RNA extracted from other pathogens that are likely co-exist with PED virus such as the salmonella, cryptosporidium and hog cholera virus. RNA templates were LAMP assayed in parallel with the RT-LAMP. The amplified products were also ran in an agarose gel electrophoresis for confirmation which showed a ladder-like DNA products in RT-LAMP and a single amplicon on RT-PCR. Real-Time PCR was also performed to confirm the extent of the RT-LAMP sensitivity in detecting the virus.

Results and Discussion

The PED RT-LAMP Test Kit

The object of the present invention is achieved by the following technical solution:
Along with the simplest nucleic extraction reagents, the kit of the present invention is limited only for the closed-tube RT-LAMP reaction solution constituted by aseptically formulated liquid reaction preparation, wherein the specifications for the 10 reaction kit. The RT-LAMP reagent components are combined in just 1 LAMP reaction mix and just 1 μL is added for the assay. Cloned plasmid construct harboring the corresponding target PCR product using the F3 and B3 is also incorporated to be included each and every assay. The storage of RT-LAMP reaction mix is -20° C or -80°C frozen transport, to avoid repeated freezing and thawing and best used within 1 month according to the storage temperature and time tested.

Analytical sensitivity of RT-LAMP

Table 2. Detection limit of RT-LAMP

Standard Dilution	Ratio	LAMP Visual*	LAMP UV **	LAMP AGE***
Std 1	Original Stock	Green	Dark	with bands
Std 2	1:10	Green	Dark	with bands
Std 3	1/100	Green	Dark	with bands
Std 4	1/1000	Green	Dark	with bands
Std 5	1/10,000	Light green	Dark	with bands
Std 6	1/100,000	Light green	Dark	with bands
Std 7	1/1,000,000	Light green	Dark	with bands
Std 8	1/10,000,000	Light green to orange	Light	
Std 9	1/100,000,000	Orange	Light	
Std 10	1/1,000,000,000	Orange	Light	
Std 11	1/10,000,000,000	Orange	Light	
Std 12	1/100,000,000,000	Orange	Light	

Table 2 shows the analytical sensitivity of RT-LAMP which can detect the lowest PED virus concentration at 0.00031 ng/µl concentration or 10-6 dilution. This can be visually proven by the green color after addition of dye, by black color under UV light (reverse) and by multiple ladder- like bands in the gel image after electrophoresis (Plate 1).

Table 3. Showing the correlation of the sensitivity test of RT-LAMP with qPCR analysis

Standard Dilution	Ratio	SQ (Starting Qty)	Melt Temperature			Cq (Quantitative cycle)
			Rep 1	Rep 2	Rep 3	
Std 1	Original Stock	305.00000	80.50	80.50	80.50	NA
Std 2	1:10	30.50000	80.50	80.50	80.50	NA
Std 3	1/100	3.05000	80.50	80.50	80.50	NA
Std 4	**1/1000**	**0.30500**	80.50	80.50	80.50	**4.97**
Std 5	1/10,000	0.03050	80.50	80.50	80.50	12.81
Std 6	1/100,000	0.00305	80.50	80.50	80.50	13.07
Std 7	1/1,000,000	0.00031	80.50	80.50	80.50	14.59
std 8	1/10,000,000					
Std 9	1/100,000,000					
Std 10	1/1,000,000,000					
Std 11	1/10,000,000,000					
Std 12	1/100,000,000,000					

qPCR shows that nucleic acid amplification started at template concentration of 0.305 ng/µl at 4.97 Cq. If the template concentration is too high (3.05 to 305.0 ng/µl), no amplification occurs (see Stds 1, 2 and 3). However, RT-LAMP amplification is not affected by high template concentration.

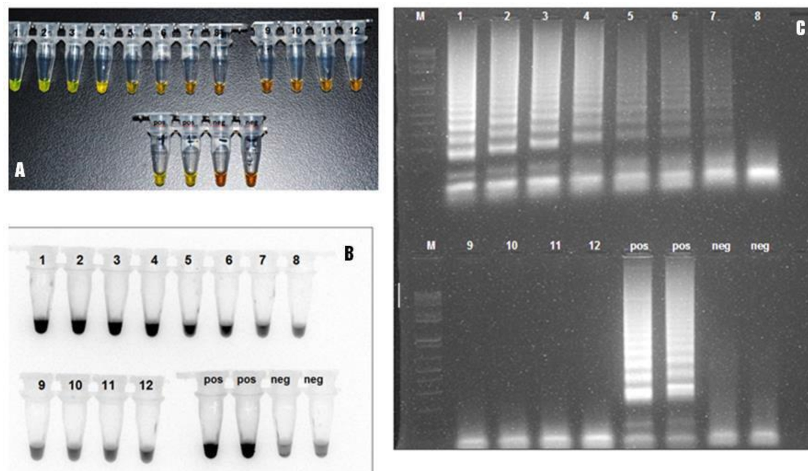

Plate 1. Positive visible results: A- visual by dye color (green), B- UV fluorescence (black) and C- agar gel electrophoresis (multiple ladder-like bands)

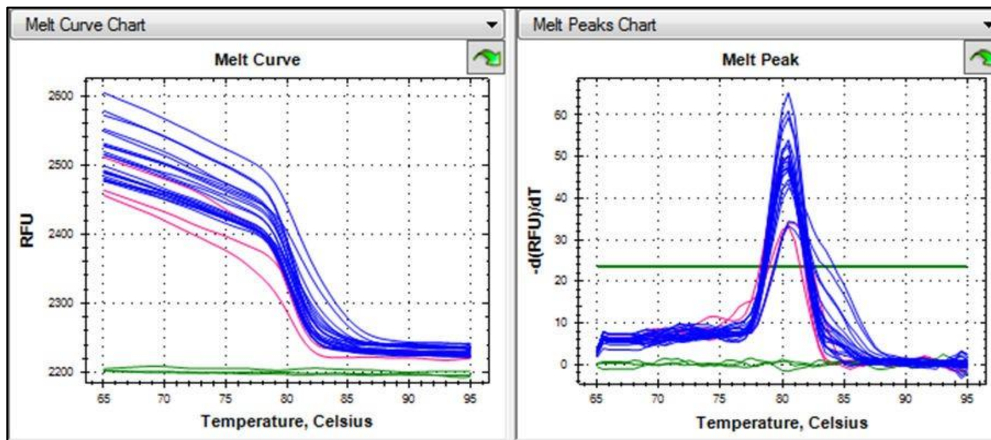

Figure 1. Melting curve and melting peak of Outer forward and reverse primers of RT-LAMP used in qPCR

Figure 1 shows that the outer forward and reverse primers of RT-LAMP used in qPCR are specific for PED virus amplification through the unified peaks at melting temperature above 80.0oC.

Analytical specificity of RT-LAMP

Analytical specificity shows that RT-LAMP is specific for the spike glycoprotein target gene of PED virus only and cannot amplify other swine gastrointestinal infections such as Salmonella Cholerasuis, Classical Swine Fever virus and Cryptosporidium pig genotype II.

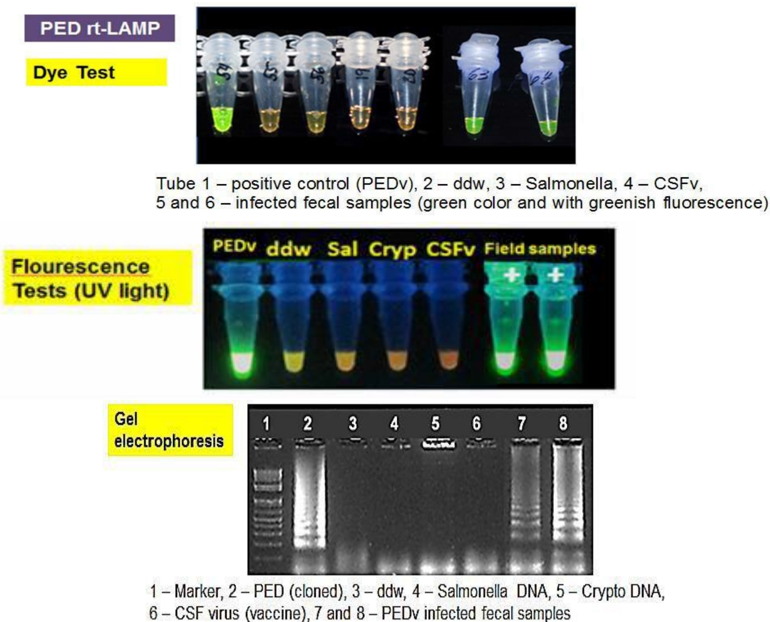

Plate 2. Visual results of RT-LAMP showing its analytical specificity by dye test, UV fluorescence and agar gel electrophoresis.

RT-PCR protocol using outer forward and backward of RT-LAMP primers

Using this cloned cDNA as reference template in RT-PCR, a PCR product with an amplicon size of 188 bp was visualized in the gel. Lanes 6 and 7 above showed two fecal DNA samples from the field that were PCR positive reactors (Plate 3).

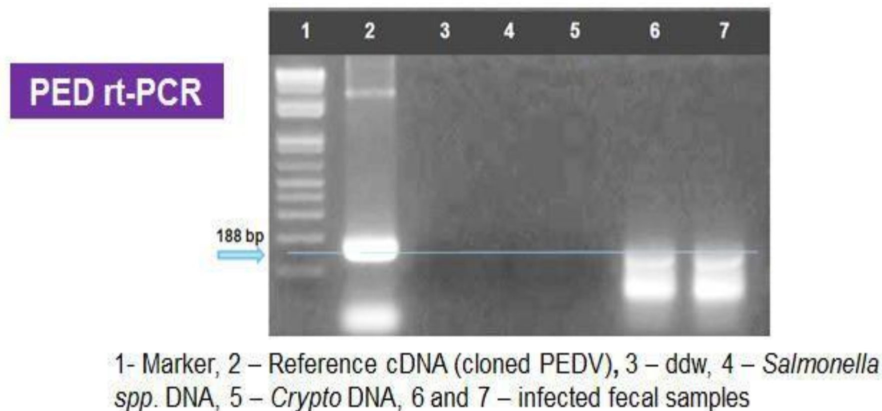

Plate 3. Gel image of the RT-PCR product of cloned PED virus showing 188 bp amplicon size.

The sensitivity of RT-LAMP was also proven when negative results were acquired using RT- PCR and positive results were obtained from RT-LAMP. To confirm that the virus can still be detected, re-PCR was performed and true enough that the band appeared. This therefore conclude that LAMP is more sensitive that PCR (Plate 4).

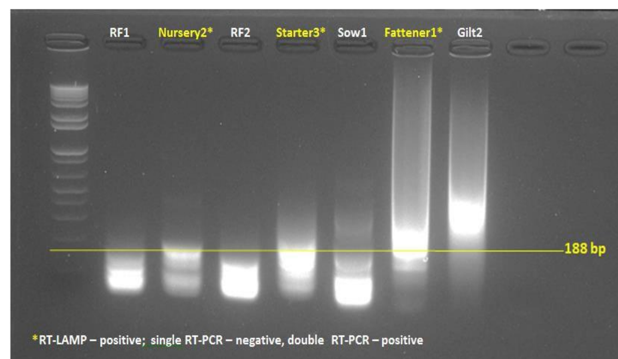

Plate 4. Re-PCR of the RT-PCR product with formerly negative for DNA bands.

Repeating the RT-PCR using the product from the first RT-PCR amplification increased the concentration of the template and bands with the expected amplicon size for PEDv were visualized (Nursery 2, Starter 3, and Fattener 3).

The identity of the RT- PCR products that fell on the expected size was

confirmed by direct sequencing. Sequencing both ends of the PCR product with the sense and antisense of the universal primers, the complete nucleotide sequence was determined. Sequencing alignment results show that the RT-PCR product corresponded with the target DNA sequence fragment of the PED virus (GenBank Accession No. KM 406181.1) using BLAST (Basic Local Alignment Search Tool) search using the NCBI database for sequence similarity proved that it matches with the PED virus Table 4.

Table 4. DNA sequences of RT-PCR products

Sequences producing significant alignments:

Select: All None Selected:0

Alignments Download GenBank Graphics Distance tree of results

Description	Max score	Total score	Query cover	E value	Ident	Accession
Porcine epidemic diarrhea virus S gene for spike protein, complete cds, strain: GDS03	228	228	98%	1e-56	98%	AB857235.1
Porcine epidemic diarrhea virus strain SC-L spike protein (S) gene, partial cds	228	228	98%	1e-56	98%	KC886306.2
Porcine epidemic diarrhea virus strain JS2008, complete genome	228	228	98%	1e-56	98%	KC210146.1
Porcine epidemic diarrhea virus isolate JS2008, complete genome	228	228	98%	1e-56	98%	KC109141.1
Porcine epidemic diarrhea virus strain SD-M, complete genome	228	228	98%	1e-56	98%	JX560761.1
Porcine epidemic diarrhea virus isolate CH/YNKM/2012 spike protein gene, complete cds	228	228	98%	1e-56	98%	JX018180.1
Porcine epidemic diarrhea virus strain CH4 spike protein (S) gene, complete cds	228	228	98%	1e-56	98%	JQ239432.1
Porcine epidemic diarrhea virus strain CH3 spike protein (S) gene, complete cds	228	228	98%	1e-56	98%	JQ239431.1
Porcine epidemic diarrhea virus strain CH/JL/2011 spike glycoprotein S2 (S) gene, complete cds	228	228	98%	1e-56	98%	JQ638924.1
Porcine epidemic diarrhea virus strain CH/BJSY/2011 spike glycoprotein S2 (S) gene, complete cds	228	228	98%	1e-56	98%	JQ638921.1
Porcine epidemic diarrhea virus strain attenuated DR13, complete genome	228	228	98%	1e-56	98%	JQ023162.1
Porcine epidemic diarrhea virus strain virulent DR13, complete genome	228	228	98%	1e-56	98%	JQ023161.1
Porcine epidemic diarrhea virus strain CH/GDQY/2011 S1 protein (S1) gene, partial cds	228	228	98%	1e-56	98%	JN601051.1
Porcine epidemic diarrhea virus strain CH/HNZZ/2011 S1 protein (S1) gene, partial cds	228	228	98%	1e-56	98%	JN601050.1
Porcine epidemic diarrhea virus strain CH/GXNN/2011 S1 protein (S1) gene, partial cds	228	228	98%	1e-56	98%	JN601049.1
Porcine epidemic diarrhea virus strain CH/GXWM/2011 S1 protein (S1) gene, partial cds	228	228	98%	1e-56	98%	JN601045.1
Porcine epidemic diarrhea virus strain CH/FJND-4/2011 S1 protein (S1) gene, partial cds	228	228	98%	1e-56	98%	JN601044.1
Porcine epidemic diarrhea virus strain CV777 spike protein (S) gene, complete cds	228	228	98%	1e-56	98%	JN599150.1
Porcine epidemic diarrhea virus strain CH/S, complete genome	228	228	98%	1e-56	98%	JN547228.1
Porcine epidemic diarrhea virus S gene for spike protein, complete cds, strain: MK	228	228	98%	1e-56	98%	AB548624.1

Cloned PEDv was developed from the field sample that was RT-PCR positive. The DNA sequence of this cloned cDNA was 98% homologous to the DNA sequence of the target gene (S gene) of the PED virus.

Findings from field validation/surveillance:

RT-LAMP prevalence for PED in Bulacan and Batangas is 65.3% (95% CI: 60.0-70.5).

Table 5. RT-LAMP and RT-PCR results from fecal samples.

		RT-PCR		
		POSITIVE	NEGATIVE	TOTAL
RT-LAMP	POSITIVE	119	90	209
	NEGATIVE	10	101	111
	TOTAL	129	191	320

Table 5 shows that RT-LAMP detected more PED positive samples than RT-PCR.

Calculating for the diagnostic sensitivity with RT-PCR as the gold standard, RT-LAMP is 100% whereas specificity is 63.64 up to 75.0%. Surveillance conducted among participating commercial and smallhold farms in Batangas and Bulacan.

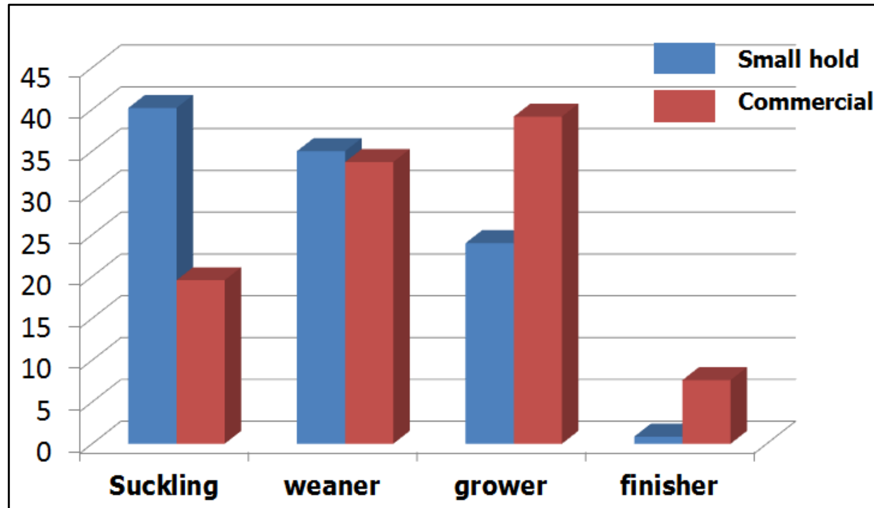

Figure 2. Percentage distribution of PED RT-LAMP reactors according to age of pigs.

Figure 2 shows that there are more PED positive pigs among growers in commercial farms. There are more PED positive pigs among sucklings in small hold farms.

RT-LAMP amplified cDNA of PED viruses in diarrheic stools even with no fulminant cases in the farm yet. There was moderate agreement between RT-PCR and RT-LAMP at 0.41 kappa coefficient. RT-LAMP amplified products were confirmed as PED virus through DNA sequencing.

Shelf-life of RT-LAMP premixes at -20°C

It was observed that the freezer of the ordinary home refrigerator can be used as cold storage for the LAMP formula. A maximum of 80 days or 2 months and 3 weeks was the shelf life of the LAMP formula where it can still amplify the nucleic acid of the complementary DNA of the virus as demonstrated in the dye, fluorescence and gel electrophoresis.

Table 6. Shelf-life of RT-LAMP premixes at -20oC storage.

Shelf Life	(-)20 °C Lab. Freezer			Home Ref. Freezer		
Day	**Dye**	**Fluo**	**Gel**	**Dye**	**Fluo**	**Gel**
1	1	1	1	1	1	1
3	1	1	1	1	1	1
7	1	1	1	1	1	1
14	1	1	1	1	1	1
21	1	1	1	1	1	1
28	0	1	1	0	1	1
35	0	1	1	0	1	1
42	1	1	1	1	1	1
49	0	0	0	1	1	1
56	1	1	0	0	0	0
63	1	1	0	1	1	1
70	1	1	1	1	1	1
77	0	0	0	0	1	0
80	1	1	1	0	1	1

Performance of RT-LAMP test kit for PED virus detection

Field validation of the PED RT-LAMP test kit was conducted on samples submitted by private veterinary practitioners of commercial farms and LGU vets from Tarlac, Pangasinan, Pampanga, Batangas and Bulacan. These samples were feces, intestines, swabbings from empty feed sacks, soiled floors and pens.

Table 7. Age of pigs detected with PED virus using RT-LAMP test kit.

*Age of Animal			TOTAL	Percent Positive
Nursing/ Starter	0-30 days	1 day to 1 month	14/37	38%
Weaner	31-70 days	1 month to 2.5 mos	2/4	50%
Grower	71 - 180 days	2.5 mos to 6 mos	33/49	67.30%
Finisher/Gilt/ Sow	above 180 days	above 6 mos	27/38	71%
		known age	76/128	59.40%
		unknown age	27/37	73%
		TOTAL	**103/165**	**62%**

The PED prevalence of 62% from fecal samples submitted by veterinary practitioners using the RT-LAMP test kit is similar to the first surveillance done during the optimization of the assay. Likewise, more grower pigs were detected to be infected with PED up to the finisher/gilt/sow. However, it was observed that all ages were affected with PED using RT-LAMP test kit.

RT-LAMP test kit can also be used for detecting the PED virus from intestines of nursing piglets that succumbed to PED (Table 8). After confirming that the intestines were PED virus positive, the veterinary practitioners used them as autogenous material for immunizing sows nearing farrowing. This stimulates the production of maternal antibodies against PED which are now sucked from the colostrum by nursing piglets. From the testimonies of these practitioners, RT- LAMP guided them to contain the spread of PED in the farm.

Table 8. Samples tested using RT-LAMP test kit.

Samples Submitted	No. Submitted	No. Positive	%
Fecal Swabs	165	103	62%
Intestines	36	36	100%
Feed Sacks	10	10	100%
Raw Feed Ingredients	5	2	40%
Floor Swabbings	4	2	50%
Farrowing Crates Swabbings	3	2	67%

Likewise, RT-LAMP test kit can also detect the PED virus from inanimate objects such as swabs of floor pens and farrowing crates, raw feed ingredients and inner surfaces of empty feed sacks (Table 8). Hence, the test kit can be a cheap but very sensitive test for monitoring the virus in the farm for early implementation of biosecurity and other control measures before an outbreak occurs.

Validation of LAMP Assays by External Evaluators

LAMP protocols were validated by two evaluators who came from the RADDL - DA-RFO3 and from the Animal Health Unit of PCC and who were not involved in the development of the LAMP protocols. They were allowed to conduct the LAMP assays themselves. After completion, they were asked to rate the assays whether they were satisfied or not. Both evaluators indicated their satisfaction towards the LAMP after evaluating the protocols of the three pathogens.

Other Accomplishments of the Research

Training Manual for the conduct of Training-Workshops on LAMP-based assays of swine gastrointestinal and respiratory infections entitled, “LAMP Training Manual: Porcine Respiratory and Intestinal Diseases” were developed (Plate 5). The manual contains the LAMP protocols and how samples are collected and prepared for the LAMP assays. Likewise, another Instruction Manual on how to use the RT-LAMP test kit for PED virus detection was also developed (Plate 6). This manual is also used for the conduct of Training-Workshops on the same topic and will be inserted in all test kits produced.

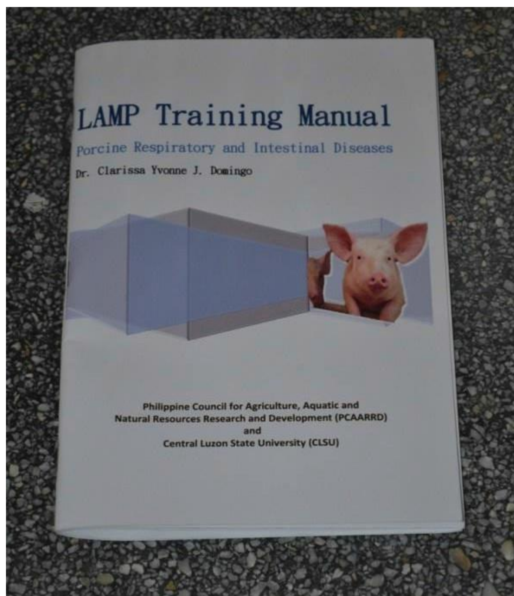

Plate 5. LAMP Training Manual for RT-LAMP

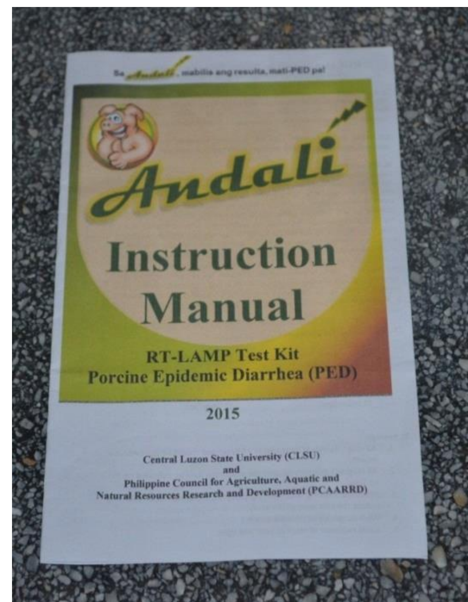

Plate 6. Instruction Manual for PED virus Detection

Fabricated heat blocks called “Lab in a Mug” were purchased from the DOST recognized Manila Health Tek, Inc. (Plate 7). With this, the PED RT-LAMP test kit developed was re-optimized using the fabricated heat blocks. The re-optimized RT-LAMP protocol was taught during the Training-Workshops conducted whereby, all participants conducted the LAMP assays using the fabricated heat blocks.

Plate 7. Fabricated heat block called "Lab in a Mug"

Trained lab personnel from the Philippine Animal Health Center of the Bureau of Animal Industry, RADDLs of different regions (I, III, IV, VIII, X and XII), SUCs that offer veterinary medicine degrees (BSU, CAVSU, BUCAF and PSAU), LGU veterinarians from Batangas and some private veterinarians of commercial hog farms in Pampanga on LAMP based assays and how to use the RT-LAMP test kit (Plates 8).

Plate 8. Techno-transfer training-workshops conducted for PED RT-LAMP test kit promotion.

Three hundred 300 RT-LAMP test kits (Plate 9) were produced with one kit having the capacity to test 10 samples.

Plate 9. PED RT-LAMP test kit

The PED RT-LAMP test kit was applied for patent application number for its appropriate primers, re-optimized LAMP protocol and assay contents to the IPOPHIL (Plate 10).

PPIP

SERAPION, JERRY C

INTELLECTUAL PROPERTY MANAGEMENT OFFICE, PHILRICE, MALIGAYA, SCIENCE CITY OF MUNOZ, NUEVA ECIJA NUE

SOA No.	:	0003220150342849
Application No.	:	12015000198
Filing Date	:	9 June 2015
Applicant	:	DOMINGO, CLARISSA YVONNE J.
Title	:	METHOD AND KIT TO DETECT PORCINE EPIDEMIC DIARRHEA VIRUS (PEDV) THROUGH REVERSE TRANSCRIPTION LOOP -MEDIATED ISOTHERMAL AMPLIFICATION (RT-LAMP)
BPI Ref. No.	:	IPPHL32150342847190

IP PHILIPPINE
PH/2015/217722
12015000198
New Application-Invention -[050]
09 Jun 2015 10:42AM (Noel)

ACKNOWLEDGEMENT

This is to acknowledge receipt of the above-identified application on June 9, 2015

This application as filed has satisfied the requirements for the grant of filing date. Processing of this application will now proceed. The Office will be sending to the applicant(s) Official Action(s) or Notice(s) to that effect.

In any dealing made before the Office, be it a query, follow-up or submission of documents, regarding this application, the applicant(s) is (are) required to indicate the APPLICATION NUMBER; FILING DATE (OR RECEIVED DATE if not Filing Date); APPLICANT; TITLE; BUREAU; DIVISION; & PERSONNEL-IN-CHARGE, if so indicated in the latest communication or Action sent by this Office. All communications or responses must be addressed only to THE DIRECTOR OF PATENTS so indicated at this address: Intellectual Property Office, Intellectual Property Center, #28 Upper Mc Kinley Road, Mc Kinley Town Center, Fort Bonifacio, Taguig City 1634, Philippines.

Plate 10. IPO application No. 12015000198 of Invention entitled, “Method and Kit to Detect Porcine Epidemic Diarrhea Virus (PEDV) Through Reverse Transcription Loop-Mediated Isothermal Amplification (RT-LAMP).

Conclusion

LAMP assay demonstrates potential and valuable means of detecting PED virus. Moreover, the simplicity of analyzing LAMP products through visual inspection makes it even more feasible for monitoring, surveillance and field diagnosis. With the results obtained from all the LAMP readings, it can be concluded that LAMP can serve as an alternative method for PCR.

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