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Development of primers for loop-mediated isothermal amplification of Philippine white spot syndrome virus isolates

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Abstract. Because there are currently no effective chemicals or drugs to completely treat bacterial and viral diseases, effective means of prevention such as early detection methods are continuously being explored and fully exhausted. Over the past few years, we have seen the development of cutting edge technologies to specifically and efficiently detect White Spot Syndrome Virus (WSSV), one of the major viruses that devastated global shrimp aquaculture. The recent discovery of loop mediated isothermal amplification (LAMP) brought about opportunities for fast diagnosis of a lot of animal diseases including humans. Here, the development of alternative LAMP primers specifically for Philippine WSSV isolates was discussed. The sensitivity of the established detection protocol was found to be at 0.3954 pg of shrimp DNA template while exhibiting high specificity to the viral target. In addition, alternative visualization techniques and comparison with other detection protocols are also discussed.
Key Words: WSSV, Philippines, shrimp, *Litopenaeus vannamei,* LAMP.

Introduction. The global shrimp aquaculture industry is worth in excess of 10 billion US dollars annually, but continues to be plagued by endemic viral diseases (Johnson et al 2008) mainly because there are currently no effective chemicals or drugs to completely treat these diseases. Thus, the ability to vaccinate shrimp and other crustaceans against specific viral diseases is of global economic and biosecurity significance. Immune-stimulation and vaccine-based approaches are likely to prove difficult in shrimp because these organisms primarily rely on innate immunity and therefore lack the ability to produce antibodies. This is the main reason why quarantining and environmental management are the most commonly used practices in combating shrimp diseases (Xiang 2001). These strategies, however, are non-specific in combating infectious diseases and cannot boost the shrimp's ability to cope with future infection even with the same pathogen (Hoffmann et al 1999). In this regard, effective means of prevention such as early detection methods are continuously being explored and fully exhausted. Various methods have been developed for the detection of White Spot Disease (WSD). These include polymerase chain reaction (PCR) (Lo et al 1996; Kim et al 1998; Tapay et al 1999), *in situ* hybridization using DNA probes (Durand et al 1996), transmission electron microscopy (TEM), histological analysis using hematoxylin and eosin–stained tissues from moribund shrimp (Wang et al 1997), and immunological methods (Lu et al 1996) and recently, loop-mediated isothermal amplification (Kono et al 2004). Distinct advantages and disadvantages in terms of sensitivity, specificity, cost and convenience vary in each

of these protocols and these have been the bases for a method's acceptance and wide usage. Of these methods, LAMP protocols pose a high potential for popular use because it is cost effective, time efficient, and has medium test complexity. Like any other DNA based detection method, however, LAMP and PCR protocols are greatly affected by primer design factors, thus making it a critical bottleneck in the initial stages of experimentation. Nonetheless, the choice of primers from existing studies or the design of new ones is based solely on the end user's judgement and requirements. A lot of PCR primers for WSSV were designed in the recent years (Lo et al 1996; Kim et al 1998; Tapay et al 1999) while the first LAMP primers were designed by Kono et al (2004). In the Philippine setting, Maralit et al (2011) revealed the limitation of the first PCR primers for WSSV and developed an alternative primer. This study, thus, aims to develop LAMP primers based on the new PCR primers that will be efficient for comparative evaluation of LAMP and PCR protocols in the Philippines.

Materials and Methods. Samples of *L. vannamei* were collected from Batangas, Zambales, Capiz and General Santos (South Cotabato) once in every location within the span of October 2009 and June 2010 (Figure 1). Two samples of each kind were preserved in ethanol for identification at the National Fisheries Resources Development Institute (Quezon City, Philippines).

Figure 1. Sampling sites where *L. vannamei* was collected.

Muscle tissues from the 1st abdominal segment of shrimps with an average size of 110 mm were placed in 1.5 mL microcentrifuge tubes with ethanol and stored until DNA extraction. DNA was extracted using Modified CTAB Extraction Method (Santos et al

2010). The tissues were minced and placed in tubes containing Cetyl Trimethyl Ammonium Bromide (CTAB) Extraction buffer (600 ul 2% CTAB pH 8.5, 30 ul of 1% Proteinase K) and finally incubated overnight in a water bath at 55°C with occasional shaking. After incubation, 600 ul of chloroform: isoamyl (3:1) solution was added to each of the sample, shaken by hand for about 3 min and then centrifuged for 5 min at 8,000 rpm. The upper aqueous supernatant was then transferred in new, marked 1.5 mL tubes, avoiding inclusion of the organic phase. Described steps of clean-up were done twice. DNA precipitation was carried out by mixing 50 ul of 3M sodium acetate and 900 ul 95% ethanol to the tubes containing the supernatant. These were then hand shaken for 3 min and placed overnight in a –20°C freezer. After precipitation, the tubes were spun in a microcentrifuge at 12,000 rpm for 10 min and then the aqueous phase was carefully pipetted out by leaving the DNA pellet at the bottom of the tube. The pellet was rinsed by adding 500 ul 70% ethanol and spun for 12,000 rpm for 5 min before the removal of ethanol. This rinsing step was done twice. Air drying was carried out right after. DNA pellets were then rehydrated in 300 ul of 1X TE buffer (1 mM EDTA, 10 mM Tris-HCl, pH 8.0). Resulting stock DNA extracts were stored in microcentrifuge tubes at -20°C.

Detection of WSSV using LAMP was carried out in a total of 25 ul reaction volume containing 2 ul (20 pmol) of each designed/published -FIP and –BIP, 1.0 ul (5 pmol) of -F3 and -B3, 12.5 ul of 2× reaction mixture (40mM Tris–HCl, 20mM KCl, 16mM MgSO4, 20mM (NH4)2SO4, 0.1% Triton-X, 1.6M Betaine and 2.8mM dNTPs each), 1 ul of target DNA and 3.5 ul of distilled water. The mixture was heated at 95^{o}C for 5 min, then chilled on ice for 2 mins. After which, 0.8 ul of Bst DNA polymerase was added. After incubation at 65^{o}C for 60 min, the reaction was terminated by heating at 80^{o}C for 10 min. The resulting LAMP products were centrifuged in a table top spinner for pellet observation and agarose gel electrophoresis (AGE) using 2% agarose for confirmation. For further visualization, 4 ul of Sybr Safe stain diluted 1000 times or Ethidium bromide diluted with PBS 1000 times was added to the LAMP products and illuminated under UV light.

Results and Discussion. New primers for LAMP experiments were designed (Table 1). The target gene of the PCR primers for WSSV by Maralit et al (2011) was also used as the target template for designing the LAMP primers. This assures that the comparison based on specificity and sensitivity is not affected by factors which relate to the primers and their target gene. Using Primer Explorer V4 software available online (at http://primerexplorer.jp/e/), five primer sets were generated using default primer design options (Figure 2).

Table 1

List of LAMP Primers Generated from Primer Explorer v4

Name	*Sequence 5' to 3'*
A-FIP	AGG GAG AAA CTG GTG ATA GCA -TTTT-ACA TTG GGT AGT AAA CAC TGG
A-BIP	CAC CAT CTT AAA GAG TTT AAC GGG C -TTTT- CTT TGC ACT AGA CAA GGA TTC
A-F3	CTC ATT AGG CTG GTC ACA T
A-B3	GGC CGA ATT CAT GGA GAT
B-FIP	CCA GTG TTT ACT ACC CAA TGT ATG T -TTTT- CAT CCA CTG TTA CAA TGT CTT
B-BIP	GTA CAG ATC AGG GAA CAT TTG CT -TTTT- ATA GAT TCA GAC CGC CCG
B-F3	GGC AAT ACT GGA GGA GGT A
B-B3	GAC AAG GAT TCA AAA TTT ACT GT
C-FIP	ACC CAA TGT ATG TGA CCA GCC -TTTT- GGA GGA GGT ACA TCC ACT
C-BIP	ACA CTG GGT ACA GAT CAG GGA A -TTTT- ATT CAG ACC GCC CGT TAA
C-F3	GAG GAG GGT ACG GCA ATA
C-B3	CAA GGA TTC AAA ATT TAC TGT GG
X-FIP	CTC TGC AGA TAC GAA TGC TTC ATA G -TTTT-CCA GCC CTT CTA AAA GAA CG
X-BIP	AGA AAC TGC CTT ACA ATT ATC TCG A -TTTT- GCG TCC AAT TCT AAT TGG TC
X-F3	AGA CGA TTT TGA GCC TAC G
X-B3	TTC CTT GTA GAG TGT CTG AG

Several factors, including the general considerations for PCR primer development, were noted. Relative positions of the 4 primers from each other, stabilities on several regions on the primers and the length of the target gene were important factors considered in selection from Primer Explorer v4 results ("A Guide to LAMP Primer Designing", 2006). Out of the primer sets generated in Figure 1, four sets were chosen for testing based on the stability of the 3' end at the F2 region, the 5' end at the F1c region, 3' end at the B2 region and the 5' end at the B1c region of the primer set components. Likewise, the presence of consecutive TT's in the sequences of the primers, especially in the inner primers, was noted. There is an initial assumption that this characteristic in the inner primers may interfere with the loop formation caused by the adaptor sequence TTTT's inserted between the regions F2 and F1c of the FIP and BIP inner primers (Figure 3).

LAMP reaction tests using four candidate primer sets (Table 1) and serial dilutions of the positive control (DNA extract from a shrimp apparently showing white spots on the carapace), showed that two of the primer sets did not perform well in producing visually efficient detections (Figure 4). Primer set X showed very low sensitivity while Primer set B returned inaccurate and inconclusive results. Only primer set A returned valid results (Figure 4, top left) but this set does not render efficient sensitivity and results showing bands that are comparable to primer set C.

Only primer set C yielded efficient results and characteristic laddering patterns in positive reactions. It was also used for test detections using two frozen shrimp samples with gross signs of WSSV. Figure 5 shows that it successfully detected WSSV in the positive controls. Moreover, pellets were easily observed by the naked eye when the resulting LAMP products were centrifuged on a table top spinner (Figure 5a).

Further visualization is possible by conventional AGE or by simply adding fluorescent dyes in the presence of DNA like SybrSafe Nucleic Acid Stain (Figure 5c) and Ethidium bromide diluted 1000 times (Figure 5d) to LAMP products and illumination with UV light. Formation of pellets after centrifugation of the LAMP products in a table top spinner is possible until 10^{-5} dilution (Figure 6). This is the main advantage of the developed LAMP reaction. However, the pellet in the 10^{-5} dilution may be so small that close observation of the tube is required.

Meanwhile, Figure 7 shows smears in a laddering pattern. This is a characteristic of a positive LAMP detection when agarose gel electrophoresis was used as visualization. This indicates the presence of WSV in the DNA extracts while the absence of these patterns indicate a negative result.

The described LAMP reaction, which is run for at least 60 minutes, detected a DNA extract that has been diluted to 10^{-5}. In agreement with the pellet results, this reaction exhibits a detection limit of 0.3954 pg, the same sensitivity described by the PCR protocol of Maralit et al (2011). A very faint smear can be seen, however, in 10^{-6} dilution but this will be difficult to assert. These results reveal that they have the same sensitivities to 0.3954 pg of DNA from a shrimp sample. Sensitivity comparable to PCR is achieved without the need for AGE; thus, greatly reducing the time spent for experimentation, the cost of chemicals and equipments and the risk of exposure to harmful UV light and Ethidium bromide. On the other hand, it is important to note that the compared primers for PCR and LAMP have identical target genes. This means that the copy number, region in the genome and other characteristics of the target templates of both methods are exactly the same. This is the reason why they have very similar detection limits. The first LAMP detection of WSV by Kono et al (2004), described a detection limit of 1 femtogram that can be achieved for at least 45 minutes. This is a rather faster protocol, although, it did not indicate pellet formation after the reaction.

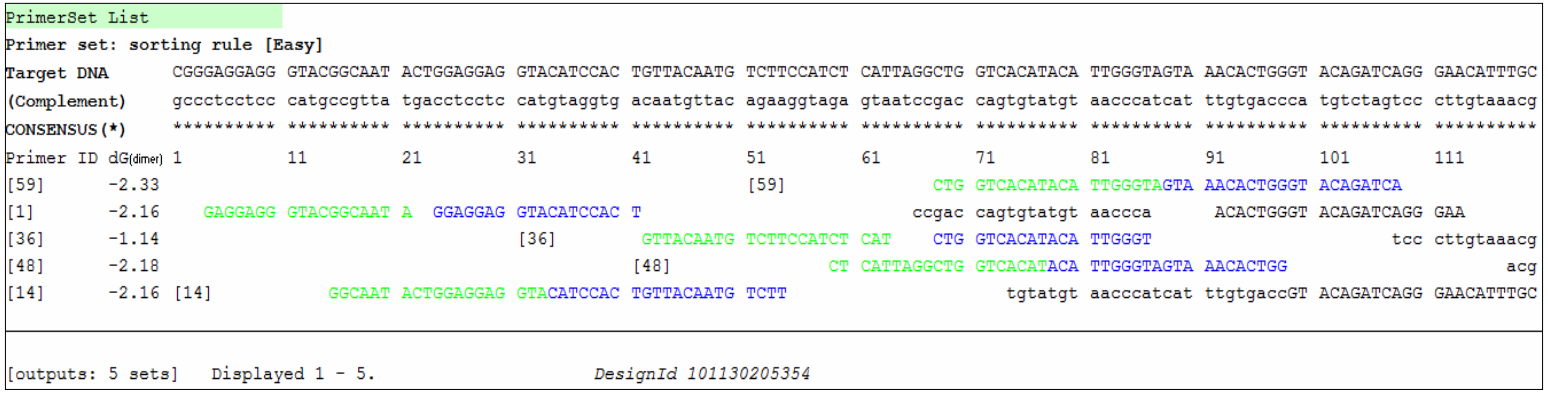

Figure 2. Result window of Generated Primers from Primer Explorer v4 showing 5 primer sets.

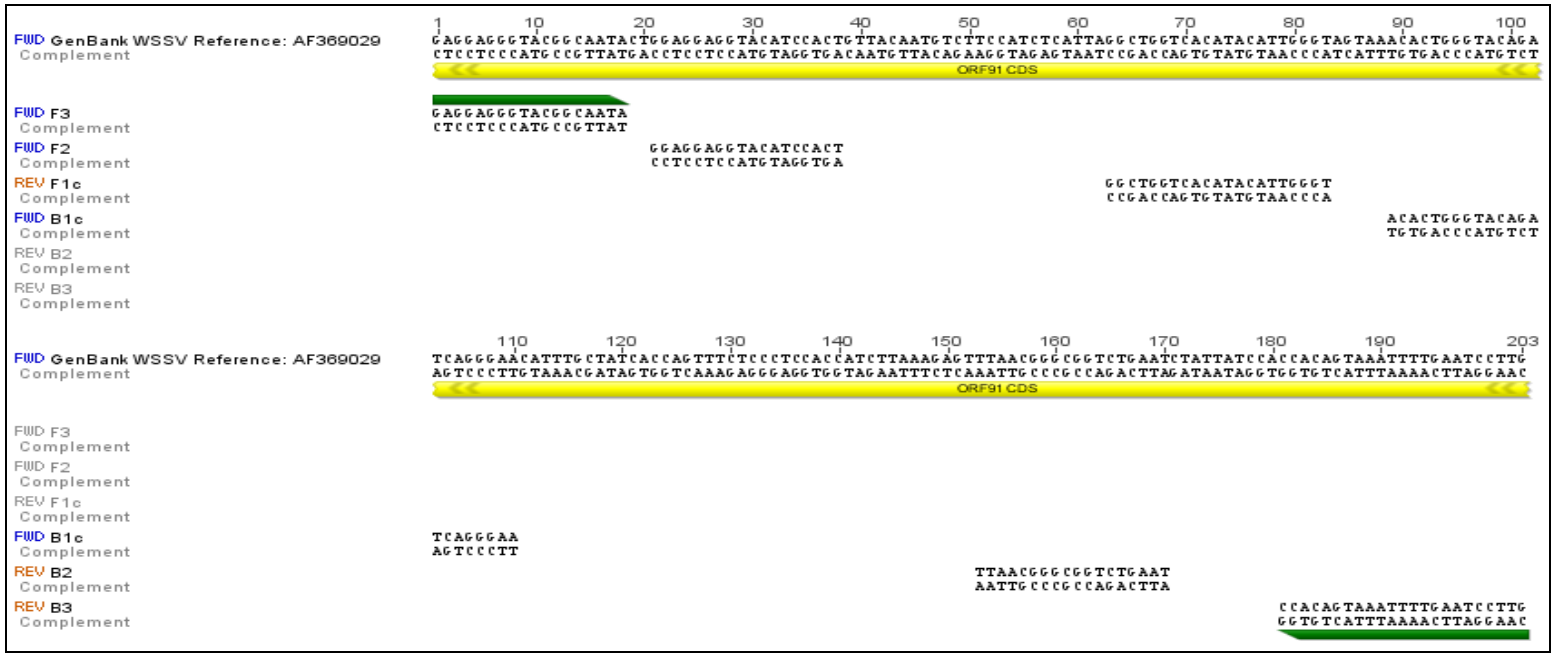

Figure 3. Components of LAMP primer set C showing their specific location in the GenBank WSSV reference (generated by Geneious 5.6 Drummond et al 2012).

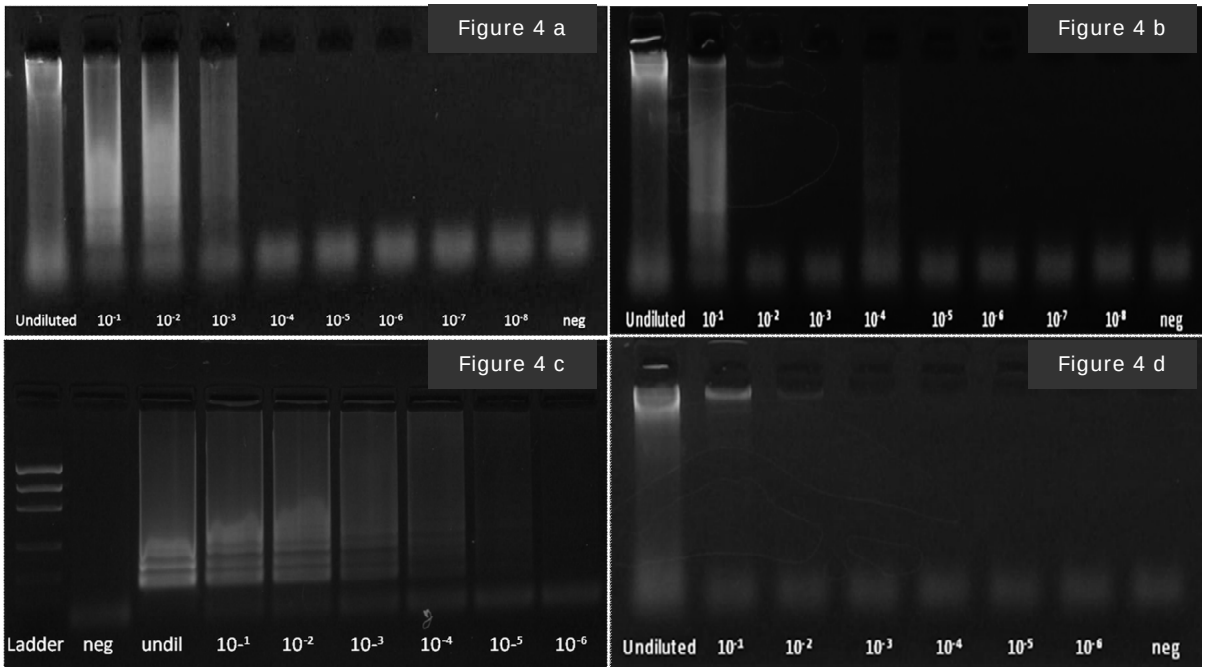

Figure 4. Serial Dilution test for LAMP Primers Sets A (4a), B (4b), C (4c), and X (4d).

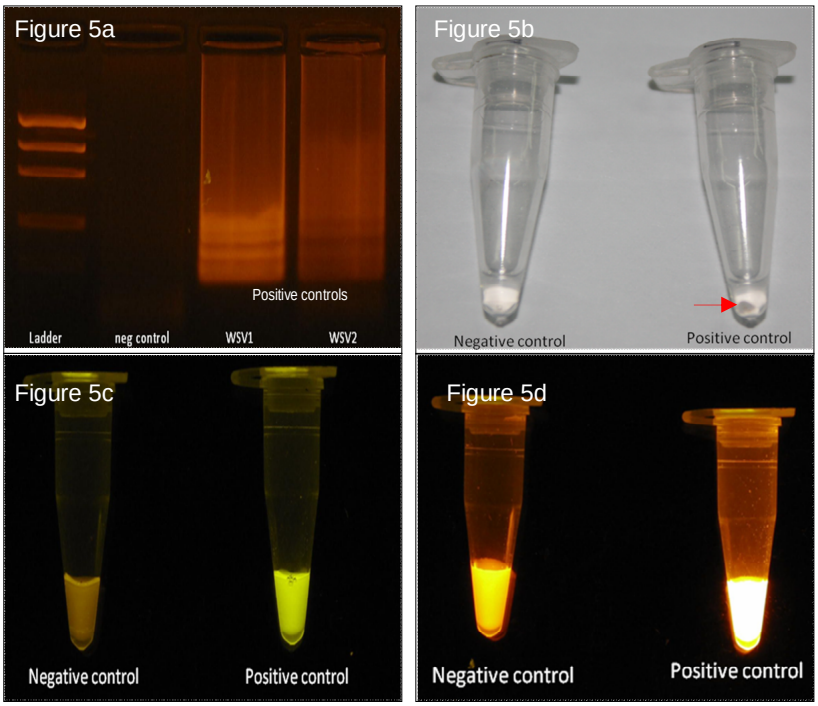

Figure 5. Visualization of LAMP Products using UV Illumination on Agarose Gel (5a), pellet formation (5b), UV illumination of LAMP products stained with SybrSafe stain (5c), and with diluted EtBr (5d).

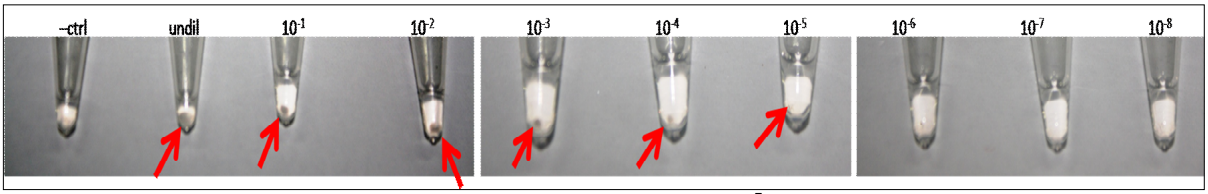

Figure 6. Pellet formation in LAMP products until 10^{-5} dilution after centrifugation.

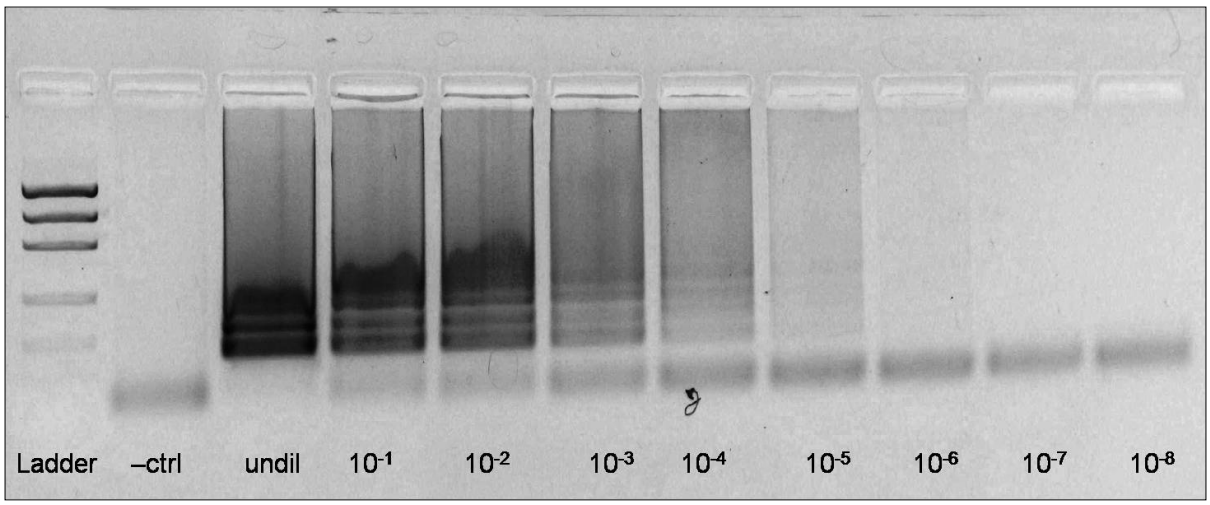

Figure 7. Test for detection limit of LAMP using 10 fold serial dilution.

Conclusion. Currently, the most widely used method in screening WSV in shrimp aquaculture is PCR, and its derivatives in the case of detection kits. In general, PCR and LAMP detection methods may be compared by the following: test complexity, specificity/sensitivity, DNA/RNA detection, quantification, multiplex application, and strain typing ability (Chen et al 2011). When it comes to these characteristics, PCR may be of greater preferential value. Both methods have the ability to detect DNA and RNA with good sensitivities but LAMP does not offer quantification of products, multiplex applications and good strain typing abilities. Moreover, the test designs of PCR, e.g. primer design, are less complex than LAMP. However, LAMP protocols pose a high potential for popular use as it does not require the stringent equipment demands of PCR. Aside from being cost effective, running time for LAMP detections, as shown in the study, is at least 1 hour compared to an average of 2 to 3 hours running time for PCR. To emphasize, AGE and UV illumination of EtBr stained products may not be necessary for visualization as pellet formation is possible. It is suggested that PCR and LAMP are similar in sensitivity and specificity, but when it comes to cost effectiveness, time efficiency and convenience in visualization, LAMP, when optimized and well established, is more preferable than PCR.

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