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9. Utilization of the loop-mediated isothermal amplification (LAMP) for the detection of pathogens in shrimp and fish aquaculture in the Philippines

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Abstract. Pathogenic diseases of fish and crustaceans pose constant threat to the sustainability and economic viability of the aquaculture industry. Early diagnosis of the disease has a crucial role in effective management in any aquaculture facility. Several biochemical and serological tests have been developed for the detection of the different pathogens of fish and crustaceans, but there are inherent constraints in logistics that make these diagnostic tools difficult to adopt during the production cycle. Molecular-based techniques particularly the conventional polymerase chain reaction (PCR) and quantitative real-time PCR have become increasingly popular in diagnostics; however, their application is limited by the high cost of equipment and reagents. Recently, a technique called loop-mediated isothermal amplification (LAMP) has been developed to detect a number of pathogens. This technique is rapid and sensitive; thus, it has been used for the detection of bacterial, viral, fungal and parasitic diseases in aquaculture. Moreover, this assay does not

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require the use of sophisticated and expensive equipment, making it to have a high potential for on-site diagnosis of pathogenic diseases. In this review, the application of LAMP for the detection of commercially-important bacterial and viral pathogens in shrimp and fish aquaculture in the Philippines is discussed.

Introduction. Aquaculture in the Philippines has a long history and involves many species and farming practices (Paclibare 2005). The bulk of production comes from the farming of seaweed, milkfish, tilapia, shrimp, carp, oyster and mussel. Aquaculture contributes significantly to the country's food security, employment and foreign exchange earnings. Aquaculture is growing much faster than capture fisheries. However, the global position of the Philippines in aquaculture production has fallen steadily over the years. The Philippines now contributes only a little over one percent of global farmed fish production compared to five percent previously.

The earliest fishponds in the Philippines were in brackishwater areas where milkfish, *Chanos chanos* are sourced from the naturally occurring fry from tidal waters. For a very long time, aquaculture in the country was synonymous with milkfish culture, specifically in brackishwater ponds, where the cultured fish relied entirely on natural food. In the early 1970s milkfish farming expanded to include culture in bamboo and net pens set in Laguna de Bay, which is the largest freshwater lake in the country. In the early 1990s milkfish culture in fish pens spread to shallow marine bays and estuaries. Soon after, milkfish culture spread to net cages, which were either fixed or floating in both freshwater and marine wáter areas. The culture of milkfish in cages was hastened by the development and marketing of commercial feeds (Yap 1999).

Shrimp has always been an incidental harvest in brackishwater ponds for milkfish. Due to a marketing campaign in the mid-1970s, black tiger shrimp, *Penaeus monodon* became popular in Japan. Before the 1980s, shrimp farming had been carried out in some coastal and brackishwater areas in the Philippines, but the real boom in production began in the mid-1980s, as wealthy families in the central Philippines began converting their sugar plantations into shrimp ponds. They deemed shrimp farming as a more profitable business venture than the farming of sugarcane. Shrimp became the top marine product export from the Philippines, earning its peak value of approximately US$ 300 000 000 in 1992. However, disease problems in the early 1990s due to luminous vibriosis and white spot syndrome caused by the white spot syndrome virus (WSSV) caused a significant drop in production. Because of this decrease in the production of tiger shrimp, the farmers shifted to the culture of another shrimp species, *Litopenaeus*

vannamei, which is thought to grow faster and are more resistant to WSSV infections. It was only recently that the government has lifted the ban on the importation of *L. vannamei* into the country as a means to revive the ailing shrimp industry in the country (Aguiba 2007).

Mozambique tilapia, *Oreochromis mossambicus* was introduced in the Philippines from Thailand in 1950. At that time, this species of fish was not well accepted by consumers due to its dark color and small size (Guerrero, 1994). In the early 1970s the introduction of Nile tilapia, *Oreochromis niloticus*, a species lighter in colour, enhanced the image of tilapia and boosted commercial production. In the late 1970s and early 1980s, commercial tilapia production was advanced by the development of technologies for the breeding of Nile tilapia in floating net enclosures and the production of Nile tilapia in floating cages with feeding. The new technologies were transferred to the private sector for evaluation. It was in 1988 when the research initiative to develop an improved strain of tilapia for low-cost sustainable aquaculture was created and resulted in the production of the Genetically Improved Farmed Tilapias (GIFT). At present, there are research initiatives toward the development of high saline-resistant strains of tilapia that can be co-cultured together with shrimp in brackishwater ponds (Jaspe et al 2007; Jaspe & Caipang 2011), as this technique produces "greenwater", which is believed to inhibit the incidence of luminous vibriosis in the ponds (Corre et al 2000).

Aside from these major aquaculture species in the Philippines, other fish species including grouper, *Epinephelus* spp., seabass, *Lates calcarifer* as well as other shrimp species such as the freshwater prawn, *Macrobrachium rosenbergii* are being tapped to be potential species for aquaculture and are likely to be considered as future export winners in the aquaculture sector. Artificial seed production and culture techniques are being developed and further refined for these different aquatic species.

Inspite of the rapid advances in aquaculture and the increasing number of aquatic species that are being utilized for aquaculture, the development of this industry is hampered by the occurrence of disease outbreaks due to pathogenic organisms. In fact, disease is recognized as one of the most serious threats to the success of commercial aquaculture (USDA, 1991). The nature of diseases affecting aquatic organisms in aquaculture systems is complex. There are more than 20 cutaneous and systemic bacterial diseases, and more than 30 viral diseases (Klontz, 1985) of commercially important finfishes.

The costs incurred in controlling and preventing diseases of fish in aquaculture systems are difficult to assess, primarily because of the virtual lack of mortality data (Klontz 1985). In this regard, there is a need for more

rapid means of diagnosis and identification of pathogens. Dixon (1985) cites the following reasons for this: first, the proper treatment of the disease can be administered promptly and may lessen the severity of a disease outbreak. Second, appropriate measures can be undertaken immediately to prevent the spread of the disease. Third, rapid detection methods may be economical in terms of time, manpower and even cost. Fourth, some of these rapid diagnostic tests may have potential for field use.

An early classification in the diagnosis of fish diseases is basically based on epidemiological, clinical, post-mortem examination, microbiological and histopathological methods (Klontz 1985; LaPatra 1996). Subsequently, the use of biochemical/serological and microscopy techniques has also been used (Dixon 1985). The serological techniques most commonly used for the identification and detection of fish antibodies among viral pathogens are the serum neutralization and immunoflourescent techniques (LaPatra 1996). Among these techniques, the ELISA has been found to be the most suitable method for early diagnosis because it facilitates identification of pathogens isolated in culture, allows accurate detection of pathogens in the diseased fish both at the laboratory and field trials, permits *in vivo* and *in vitro* studies on the amount of immune response present, and facilitates antibody surveys as a response to the invading pathogen (Robertson 1981).

With the advent of rapid advances in the field of molecular biology, a surge in the growth of new methods for diagnosing fish diseases has also been observed. In particular, the use of the polymerase chain reaction has been developed to ascertain the presence of the viral and bacterial pathogens in fish and shellfish (Austin, 1998). Other methods that have been developed include restriction enzyme digestion, probe hybridization, and nucleotide sequencing (Cunningham, 2002). Many of these molecular techniques are potentially faster and more sensitive in identifying fish diseases than the traditional methods such as serology, cell culture, and histology. In addition, the use of molecular biology has the advantage of readily detecting genetic variations in pathogens that denote subspecies or strains (Cunningham, 2002).

These PCR methods, however require either high precision instruments for amplification or elaborate methods for detection of the amplified products (Parida et al 2008). In addition, these methods are oftentimes cumbersome to adapt for routine diagnostics in the field. PCR has several intrinsic disadvantages, such as the requirement for thermal cycling, and time-consuming post-PCR analysis, that could potentially result in laboratory contamination. The comparative features of the different methods of detecting pathogens in shrimp and fish are shown in Table 1.

Table 1. Comparative features of the various pathogen detection techniques in shrimp and fish.

Features	Microscopy/Histology	Conventional PCR	Real-time PCR	LAMP
Early confirmatory diagnosis	Maybe difficult	Possible	Possible	Possible
Quantitative Detection	Difficult	Difficult	Possible	Possible
Requirement of equipment	Cheap and readily available	Expensive	Expensive	Cheap and readily available
Application in field	Difficult	Difficult	Difficult	Possible
Visual detection	Yes	No	No	Yes
Risk of contamination	Low	High	High	High
Ease of operation	Easy	Average	Average to Difficult	Easy
Analysis of multiple samples in a single reaction	Possible	Possible	Possible	Difficult

Loop-mediated isothermal amplification (LAMP): principles and methodology. Loop-mediated isothermal amplification is a sensitive strand displacement technique (Notomi et al 2000). This method amplifies target DNA from a few copies to 109 copies in less than an hour under isothermal conditions. It is an offshoot of the basic strand displacement techniques which have been described thoroughly (Notomi et al. 2000). Briefly, four highly specific primers are constructed from the target DNA, one set of primers anneal to the target region one after the other on the same strand and the primer which anneals at the later stage displaces the strand formed by the first primer with the help of Bst DNA polymerase, which has a strand displacement activity. This takes place on both strands and the primers are designed such that loops are formed. The reaction is carried out under isothermal conditions as denaturation of the strand takes place by strand displacement. The reactions produce a series of stem-loop DNAs with various lengths. The four primers hybridize against six distinct sequences in the target DNA making it highly specific.

The design of highly sensitive and specific primers is crucial for performing the LAMP assay. The primer set for LAMP amplification includes a set of six primers comprising two outer, two internal and two loop primers that recognize eight distinct regions on the target sequence. The two outer primers are described as forward outer primer (F3) and backward outer primer (B3). They have a role in strand displacement during the non-cyclic step. The internal primers are known as forward internal primer (FIP) and backward

internal primer (BIP). These primers have both sense and antisense sequences in such a way that they aid in the formation of a loop. Further, two loop primers, the forward loop primer (FLP) and backward loop primer (BLP) are designed to accelerate the amplification reaction (Nagamine et al 2002). By using loop primers, the time required during the assay is reduced by half, making it a more efficient tool in disease diagnostic applications.

The LAMP reaction is carried out using a Bst DNA polymerase along with dNTPs and reaction buffer. The reaction proceeds at isothermal conditions for 1 h or less depending on the efficiency of the developed primers and the template DNA. The template DNA for LAMP is denatured for 5 min at 95°C before setting up the LAMP reaction for isothermal amplification (Notomi et al. 2000). However, studies have shown that non-denatured templates can also be used directly for LAMP-mediated detection (Nagamine et al 2001). The LAMP reaction is carried out at 60–65°C for 45–60 min and the reaction is terminated at 80°C for 2 min. This assay does not need thermocyclers. Because amplification is done under isothermal conditions, only a water bath or heating block is needed to maintain the desired temperature.

On the other hand, the amplification of RNA template is accomplished through reverse transcription-loop-mediated isothermal amplification (RT-LAMP) assay by a reverse transcription step in addition to the Bst DNA polymerase (Parida et al 2008). RT-LAMP method can synthesize cDNA molecules from template RNA and apply LAMP technology to amplify and detect the target sequence. As the template is an RNA sample, in addition to the reagents of DNA amplification, a reverse transcriptase (RTase) is added to the reaction mixture. After mixing and incubating the samples at a constant temperature between 60–65°C, amplification and detection can be carried out in a single step. Figure 1 shows the process of carrying out LAMP assays to detect various pathogens in shrimp and fish.

Monitoring of the LAMP assay can be carried out with naked eye inspection either in the form of visual turbidity or visual fluorescence. The turbidity of magnesium pyrophosphate can be visually observed. Following amplification, the tubes can be inspected for white turbidity through naked eye after a pulse spin to deposit the precipitate in the bottom of the tube. The tube containing the amplified products can also be better visualized in the presence of fluorescent intercalating dye, *e.g.*, ethidium bromide, SYBR Green I or calcein followed by illuminating with a UV lamp. The visual inspection for amplification is done through observation of colour change following addition of SYBR Green I to the tube. In positive samples, the original orange color of the dye will change into green that can be judged under natural light or under UV light. In case there is no amplification, the original orange color of the dye will be retained.

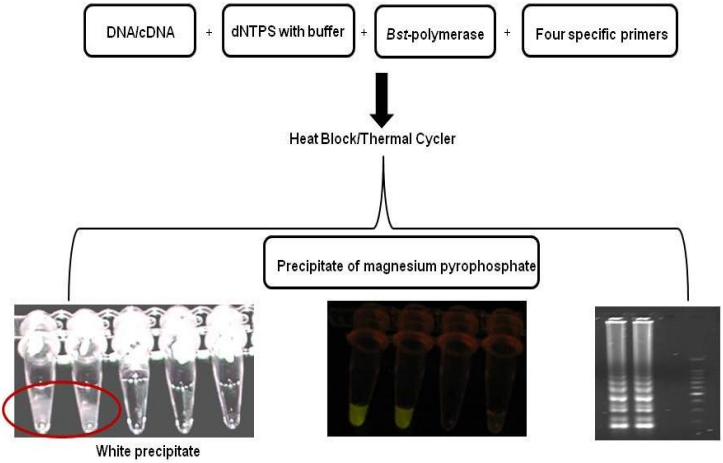

Figure 1. Steps involved during LAMP assay and methods of detecting LAMP products.

Calcein is another chelating fluorescent detection reagent that can be used in the reaction mixture prior to amplification. Calcein initially combines with manganese ions to achieve a quenching effect. The amplification generates the by-product, pyrophosphate ions, which bind and remove manganese ions from calcein to irradiate fluorescence. The fluorescence is further intensified as calcein combines with magnesium ions. The presence of fluorescence indicates the presence of target gene and visual detection is achieved without opening the tube, thus preventing carry-over contamination with post-amplification products.

Another way of monitoring the LAMP assay is through the use of agarose gel electrophoresis. Following incubation at 63°C for 30 min, an aliquot of LAMP products are electrophoresed on a 3% agarose gel in Tris-borate buffer followed by staining with ethidium bromide and visualization on a UV transilluminator. On agarose gel analysis, the LAMP amplicons show a ladder-like pattern in contrast to a single band as observed in PCR. This is due to the cauliflower-like structures with multiple loops formed by annealing between alternately inverted repeats of the target in the same strand.

Real-time monitoring of LAMP amplification can be accomplished through spectrophotometric analysis with the help of loop amp real-time turbidimeter that records the turbidity (Parida et al 2008). The turbidimeter is relatively inexpensive as compared to the real-time PCR machine. Turbidity

is the unique phenomenon associated with LAMP amplification and it is attributed to the higher amplification efficiency of LAMP reaction.

The primary characteristics of the LAMP are its ability to amplify nucleic acid under isothermal conditions in the range of 65^{o}C; as a result it allows the use of simple and cost-effective reaction equipment. Both amplification and detection of gene can be completed in a single step, by incubating the mixture of gene sample, primers, DNA polymerase with strand displacement activity and substrates at a constant temperature. Another characteristic is that LAMP has high specificity and high amplification efficiency. It has a high specificity because the assay can amplify a specific gene and can discriminate a single nucleotide difference. The high amplification efficiency of LAMP is attributed to the reduction in time loss due to thermal change because of its isothermal reaction. The reaction can be conducted under optimal temperature of the enzyme and the inhibition reaction at the later stage of amplification is less likely to occur compared with the PCR. It was observed that when nucleic acid is amplified by the LAMP method, the turbidity derived from the precipitate is produced according to the progress of the reaction and thus making it ideal for easy monitoring visually through naked eye.

The high sensitivity of the LAMP system makes it susceptible to false positives because of carry-over or cross-contamination. Amplification and detection should therefore be carried out in separate working areas. As positive reactions are seen as a smear, together with some bands of low molecular weight, and are not seen as a single band as in PCR, the specificity of amplification should be thoroughly validated to ensure that the primers only amplify the target sequence of the specific pathogen. Alternatively, the specificity can be determined by cutting the amplified products using restriction enzymes specific to the target sequence. Detection of two or more pathogens in a single reaction, as in multiplex PCR or nested PCR, may be difficult using LAMP.

Application of LAMP for detection of pathogens in Philippine aquaculture. The use of the LAMP assays have been used to detect some major viral and bacterial pathogens in shrimp and fish (Savan et al 2005). These LAMP assays have been optimized for the sensitive and rapid detection of commerically important viral pathogens in shrimp including *Penaeus monodon*, *Litopenaeus vannamei* and *Macrobrachium rosenbergii*. LAMP was also used to detect a bacterial pathogen in sea bass, Lates calcarifer.

LAMP assay was optimized to detect the *Penaeus monodon*-type baculovirus (MBV) in hatchery-reared postlarvae of shrimp, P. monodon (Caipang et al., 2011b). MBV has a wide geographical range, but this virus does not pose a serious threat to the shrimp culture industry as long as proper

management of the aquaculture facility is done to prevent secondary infections (Flegel 2006). MBV infections result in the stunting of the growth of shrimp, thus, a reduction in the price during harvest. Hence, it is important that efficient screening methods are available to detect the presence of this virus during the early stages of infection in the shrimp postlarvae before they are stocked in the ponds. Previously, a genomic DNA fragment of the MBV was sequenced and showed differences between the Philippine isolate with the other geographical isolates of this virus (Caipang et al 2011a). This sequence was used to design LAMP primers for the detection of a Philippine isolate of MBV. Results showed that the LAMP assay for the detection of MBV has an optimum reaction time and temperature at 60 min and 63^{o}C, respectively. The assay was highly specific for MBV and did show cross-reactions with other shrimp viruses. The assay can detect the virus up to a concentration of 10 pg of viral DNA ml^{-1} or 10 fg of the genomic DNA per LAMP reaction. The assay was also 10 times more sensitive than conventional PCR in detecting MBV from infected shrimp postlarvae and detected the virus from naturally-infected shrimp, indicating its potential to be used in the field.

The infectious hypodermal and hematopoietic necrosis virus (IHHNV) is another shrimp virus that can be detected using LAMP. IHHNV is a single-stranded DNA virus that infects both wild and cultured shrimps. This virus infects two important shrimp species, *Litopenaeus stylirostris* and *Litopenaeus vannamei*. *L. stylirostris* are highly susceptible to this virus and during a disease outbreak as much as 90% mortality of the cultured stock is observed (Lightner et al 1983). On the other hand, this virus is not lethal to *L. vannamei*, but can lead to growth reduction and cuticular deformities in the affected shrimp (Kalagayan et al 1991), that could result in decreased market value of the harvested shrimp (Lightner & Redman 1998). In the Philippines, IHHNV has been detected from both *Penaeus monodon* and *L.vannamei* based on the sequences of the IHHNV capsid protein gene that have been deposited in the public database (Genbank, http://www.ncbi.nlm.nih.gov/nucleotide/). A partial sequence of the capsid protein gene of this virus was identified from an infected *P. monodon* postlarvae (Caipang et al., 2011c) and the sequence was used to design primers for the LAMP assay to detect this viral pathogen (Caipang et al., 2012a). The optimum condition of the LAMP assay for the detection of IHHNV was conducted at an incubation time of 1 h at 63^{o}C. It was highly specific for IHHNV and the limit of detection of the virus was at 10 pg of the template DNA/mL or 10 fg of the template DNA per LAMP reaction. The assay was 10 times more sensitive than conventional PCR in detecting the viral pathogen from shrimp post larvae that were naturally infected with IHHNV.

Early detection of the white spot syndrome virus (WSSV) has also been made possible through the use of the LAMP assay. WSSV is one of the devastating viruses that affected the shrimp culture industry. This virus infects other crustaceans that are found in both freshwater and marine environment (Lo *et al*., 1996). It affects the entire life stages of the shrimp (Karunasagar *et al*., 1997) and during a disease outbreak, mortalities could reach up to 100% in a short period of time (Takahashi *et al*., 1994). Kono et al (2004) were the first to develop a LAMP assay for the detection of WSSV in cultured shrimp. Recently, Maralit et al (2011) tested the different PCR and LAMP assays in detecting WSSV in shrimp in various localities in the Philippines. They found limitations in the earlier LAMP primers when detecting some WSSV-positive shrimp samples. Thus, they designed several LAMP primers and tested these primer sets for the detection of WSSV from *L. vannamei* that were obtained from various sampling sites in the Philippines. The new set of LAMP primers was efficient and highly specific for WSSV detection (Maralit et al 2012). It has a limit of detecting the virus at approximately 0.4 pg of WSSV-infected shrimp DNA. Subsequently, Caipang et al (2012b) optimized a LAMP assay for WSSV detection in hatchery-reared *P. monodon* postlarvae using another set of primers. The optimum condition for this assay was carried out for 60 min at an incubation temperature of 65^{o}C. The assay also was highly specific for WSSV. The limit of detection of the WSSV in the shrimp postlarvae using the LAMP assay was 5 pg of DNA ml^{-1} or 5 fg of the genomic DNA per LAMP reaction. LAMP was 10 times more sensitive than conventional PCR in detecting the virus from infected samples and detected the virus from naturally-infected postlarvae.

There are limited studies on the development of LAMP assays for the detection of pathogens in cultured fish in the Philippines. To date, there is only a LAMP assay in detecting *Vibrio harveyi*, a bacterial pathogen of Asian seabass, *Lates calcarifer* in the country. In earlier studies done in other countries, LAMP assays have been developed detect various species of *Vibrio* in the aquatic environnment, including *V. alginolyticus* (Cai et al 2010), *V. nigripulchritudo* (Fall et al 2011) and *V. parahaemolyticus* (Sun et al 2012). This assay was also used to detect *V. harveyi* in marine shellfish using *toxR* as the target gene (Cao et al 2010). In these studies, the LAMP method was faster, easier to perform and had higher specificity and sensitivity than conventional PCR assays. The LAMP assay for the detection of *V. harveyi* in seabass was developed by Caipang et al (2012 c). They designed primers targeting the dnaJ gene (Caipang et al 2011d) of the bacterium. It was optimized at an incubation time of 1 h at 63^{o}C. The assay was highly specific for *V. harveyi* and did not cross-react with other bacterial pathogens of fish. However, the assay was able to detect *V. harveyi* that was isolated from

infected shrimps. The limit of detection of the LAMP assay was 40 pg of DNA mL^{-1} or 40 fg of the genomic DNA per LAMP reaction and was 10 times more sensitive than conventional PCR in detecting the bacterial pathogen from infected samples. The LAMP products can be quantified spectrophotometrically at 590 nm using hydroxynaphthol blue (HNB) dye and showed positive correlation with the amount of the pathogen.

Table 2 shows the primers that were used in developing LAMP assays for the detection of the different pathogens in shrimp and fish aquaculture in the Philippines.

Table 2. Primers for the LAMP assays in detecting viral and bacterial pathogens of shrimp and fish.

Primer Name	*Sequence (5′ --- 3′)*	*Source*
A. MBV		(Caipang et al 2011b)
1. F3	TGTTCTATACATTTTGCAAAGC	
2. B3	AAAGGAGTGCAGATCTTGA	
3. FIP	AAGGTCAGCAAAAAACACTCAATTTT-TTCCTCTACTGATATGGTATCAATG	
4. BIP	AAGAATCACCGGGATCCTTCATTTTA-AATCTATATAGCGTTAACACGT	
B. IHHNV		
1. F3	TCAATACATGTTACTTTCCAAAC	(Caipang et al 2012a)
2. B3	ATCCGTAGGTTTTCATCATTG	
3. FIP	TGGGAGGCAGTATAAATTTTTTCCGT-TTTGATACTTCGAATTCGACGCT	
4. BIP	TATCTCTATGGTCTGAAGAGCAG-TTTTTTTACCATTTTATATCGCTGTGTTTC	
C. WSSV		
Primer Set 1		(Maralit et al 2012)
1. F3	GAGGAGGGTACGGCAATA	
2. B3	CAAGGATTCAAAATTTACTGTGG	
3. FIP	ACCCAATGTATGTGACCAGCCTTTT-GGAGGAGGTACATCCACT	
4. BIP	ACACTGGGTACAGATCAGGGA ATT-TTATTCAG ACC GCC CGT TAA	
Primer Set 2		(Caipang et al 2012b)
1. F3	ATTTCTTTGCTCGAGGCC	
2. B3	CTAGATATTCCTTCTTCTTGTGTT	
3. FIP	GGTACTGCATTCTCTAGAGCAAAATTTT-CGGCTGGAGATTTTTTCC	
4. BIP	ATTGAAAATCGTGCAAGAACTACGTT-TTGGTAAAATATTGGTCCAGTCTCA	
D. Vibrio harveyi		(Caipang et al 2012c)
1. F3	GCGATATCTTTGGCGGTGG	
2. B3	TTGGCGCATTTGAACTTGAC	
3. FIP	ACGAACGGCTTCTTCTAGCGACTT-TTTCAGCAACGTGCACAACG	
4. BIP	GTGATACCTGTGACGGTAGCGGTT-TTGTGACCATGACAGGTTCCAC	

Concluding remarks. The aquaculture industry is rapidly expanding in order to meet the growing food demands of the increasing population. However, the sustainability of this industry is threatened by a myriad of factors and one of them is the occurrence of disease outbreaks in the aquaculture site. Therefore, sensitive and rapid detection of the infectious agents must be identified prior to the onset of mass mortality. By knowing the cause of the infection at an early stage, the aquaculturist can effectively devise proper husbandry procedures that could mitigate losses due to mortality of the cultured stock. In this paper, we have discussed the use of the LAMP technology for the diagnosis of infectious diseases in shrimp and fish and the recent application of this detection method in Philippine aquaculture. The main reason for the development of this technology is to provide a rapid, cost-effective, and accurate diagnosis for patients in any situation. Such a technology will surely contribute to quality of life now and in future for patients in both developed countries and locations where diseases are endemic.

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