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[1] Prevention and Treatment of Diseases Caused By Fish Pathogens

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2. DIAGNOSIS OF DISEASES

2.1. Diagnosis -Antiserum Detection

Tae-Sung Jung

2.1.1. Synopis

In the advent of readily available diagnostic kits, coupled with the rapid advancement in the field of genetic manipulation, the application of antibody-based diagnosis seems to lose its significance (Cunningham, 2002). Not to mention the difficulty in producing specific antibodies and the time needed for it to be usable. However, it remains indispensable and essential in understanding immune response mechanisms and development of effective vaccines and has high efficiency in the aspect of rapid diagnosis without the need for any complicated machines and kits (Adams and Thompson, 2006). Here, some useful applications of antibody-based diagnosis will be introduced, from basic agglutination to immunochromatography assay.

2.1.2. Introduction

Animals immunized by antigens (or immunogens) produce antibodies in response to proteins or other molecules recognized as foreign by their immune system (Tizard, 2010). Of course, there are several factors to be considered in terms of immune intensity. Firstly, immunogens are composed of foreign proteins, carbohydrates, lipid, enzymes, virus and bacteria. Good immunogens are high molecular weight and highly purified proteins recognized as foreign body which has high digestibility but low solubility. Secondly, immunogenicity differs depending on the injection site, antigen processing and animals immunized. Particulate antigens are normally injected intravenously, but protein antigens and bacterial carbohydrates are immunized through I.M., I.D. or S.C. after mixing with proper adjuvant to enhance immune response without generating unwanted antibodies (Ellis, 1988). There is no rule of thumb on how many times the animal needs to be injected and how much antigen to be injected. In order to achieve high antibody response, it is usual to conduct repeated exposure to the immunogen, so a series of injections at regular intervals is useful to produce both high levels of antibody and antibodies of high affinity.

Recently, animal welfare issues are getting much attention, as an alternative, chicken IgY was made available (Nho et al, 2009). It has the advantage of producing large amounts of specific antibodies without sacrificing experimental animals and the IgY produced is more or less phylogenetically distant from others thus reducing non-specific reaction.

Concerning monoclonal antibodies (mAbs), even though there are several advanced methods developed in producing mAbs, it basically needs animals such as mice or rats to immunize antigens, fuse, and continue mass production depending on the purpose. It is well known that mAbs have high specificity compared with polyclonal antibodies, which makes it possible to differentiate between false positive and positive or negative results. Recently, mAbs have been applied in developing lateral imunochromatography assay for convenient and rapid diagnosis of some viral diseases (Lipman et al, 2005).

2.1.3. Diagnosis

2.1.3.1. Agglutination Reaction

Agglutination is an antigen-antibody reaction especially between particulate antigens and its specific or cross reactive antibodies, easily observed by the clumping of these particles. The reaction occurs quickly and is easy to produce making it a very useful tool in diagnosis. This reaction is also applied to detect unknown antigen with known antibody (direct method) or vice versa, unknown antibody with known antigen, usually to check for bacterial infection. An improved version (indirect method), wherein soluble antigens or antibodies are used to coat latex, bentonite, colloidon and bacteria to detect its antibody from sera or antigens from tissues etc., was also developed. There are several methods for agglutination depending on which tool used, such as plate agglutination, tube agglutination, and 96 (u or v type) agglutination tests. In the case of viral hemagglutination and hemagglutination inhibition tests, these are very limited in the aquaculture field but are applied for myxovirus, paramyxovirus, arbovirus and poxvirus (Roberson, 1993).

2.1.3.2. Fluorescent Antibody Test or Immunofluorescence Antibody Test

Fluorescent antibody test basically applies the same principle as the ones above. It is carried out using a fluorescence microscope having a different light source, usually a mercury lamp and using distinct wavelengths, which hits antibodies attached to luorescent dyes and visualized through specific color associated with antigen and antibody (Marja and Richard, 2006). It is a fairly easy technique and only needs a fluorescence microscope, which is why it is widely used in the field of diagnosis especially for viral infection in cells and tissues. A good example is immunohistochemistry that allows for the detection of the location of antibodies. In the introduction of new fluorophores and microscopes, especially the use of epifluorescence microscope and the confocal microscope, this technique has advanced considerably from the conventional IFA test. This test has several advantages: the capacity to use mAbs and polyclonals (Anderson, 1993); high sensitivity and specificity; can be applied for bacteria determination; can be used to label single cells; and it allows the use of different types of fluorescent-labeled antibodies to observe multiple cell types in one sample. However, it can give cross reactivity when polyclonal antibodies are used, so careful analysis of the results should be made to avoid false positive or negative conclusions. The application of this test has two different methods, direct and indirect.

2.1.4. Direct Method

Direct immunofluorescence uses a single antibody combined chemically with a fluorophore. The antibody recognizes the target molecule and binds to it, and the fluorophore gives specific color to examine the sample. Because it only involves one step, it can reduce the number of steps in the staining procedure and can reduce background signal by avoiding some issues with antibody cross-reactivity or non-specificity. However, it is very difficult to bind the fluorescent molecules with antibody and is known to be less sensitive than indirect immunofluorescence.

2.1.5. Indirect Method

Indirect immunofluorescence uses two antibodies, the primary antibody specifically binds to a target molecule, and the secondary antibody, carrying the fluorophore, recognizes the primary antibody and binds to it. The secondary antibody recognizes the constant region on the first antibody, which allows the indirect method to use a variety of secondary antibodies which are commercially available.

2.1.6. Flow Cytometry Analysis

Recently, cytometry analysis to differentiate cells based on phenotypes is gaining more importance. This technique is highly similar to IFA test in terms of antigen and antibody reaction (Thuvander et al, 1992). It uses laser light to hit the fluorescent molecules attached to antibodies giving rise to signals that can be detected by cytometry. There are direct and indirect methods depending on the antibodies attached on fluorescent molecules.

2.1.7. Virus Neutralization Test

Neutralization of a virus is defined as the loss of infectivity through reaction of the virus with specific antibody. Virus and serum are mixed under appropriate conditions then inoculated into cell cultures, eggs or animals. The presence of un-neutralized virus can be detected by reactions such as cytopathic effect (CPE), haemadsorption/haemagglutination, and plaque formation. The loss of infectivity is brought about by interference of the bound Ab with any one of the steps leading to the release of the viral genome into the host cells. Even though this method is labor intensive and dependent on cell cultures, it is highly sensitive and specific (Kim et al, 2011). Moreover, this test is very useful to recognize the immune response after vaccination for a virus in mammals.

2.1.8. Enzyme-Linked Immunosorbent Assay (ELISA)

ELISA, is a technique that is extensively used for its rapidity, high sensitivity and high specificity even for small amounts of test samples (Alexandra, 2006). It is performed in 96-well plates known as ELISA plate which permits high throughput results. In order to perform ELISA test, first, either the antigens or antibodies need to be coated to allow them to stick to a polyvinyl plate, and then washed to prevent nonspecific reaction of unbound antigens or antibodies. The corresponding secondary antigen or antibody is then added which reacts with the antigens or antibodies fixed on the plate. An enzyme is tagged on the second antigen or antibody and this enzyme reacts with a suitable substrate when it is added, producing a color which is measurable as the quality or quantity of antigens or antibodies present in the given sample and thereby identified. When the enzyme reaction is complete, the entire plate is placed into a plate reader which measures the optical density (i.e. the amount of colored product) for each well. The intensity of the color produced is proportional to the amount present in the sample. Qualitative ELISA simply evaluates whether the results are positive or negative. Quantitative ELISA meanwhile, measures the optical densities or fluorescent units of the sample that are compared with a standard curve to determine the quantity. ELISA can be used to measure

serum antibody concentration, determine antigens and measure some toxin or allergens. ELISA can be used in different ways depending on the purpose. Direct ELISA uses only one set of antigens and one set of antibodies to react: Ag + Ab-E →Reaction color. Indirect ELISA uses additional antibodies added in the reaction: Ag or Ab + Ab or Ag + Ab-E → Reaction color. Sandwich ELISA is a kind of indirect ELISA, the only difference is that antigen is present between two antibodies: Ab + Ag + Ab-E→ Reaction color. Competitive ELISA is a slight modification of direct, indirect and sandwich ELISA. One more substance is added to compete with Ab or Ag to bind to the already added Ag or Ab during the reaction. The addition of this competitor substance prevents unnecessary binding of Ab or Ag, thereby promoting greater affinity between Ag or Ab. The process remains the same with other ELISA.

2.1.9. Immunochromatography Assay

Lateral flow tests also known as Lateral Flow Immunochromatographic Assays are simple devices aimed to identify the presence (or absence) of target antigens in the sample and doing so without specialized and costly equipments (Oh et al, 2006). The devices are initially developed for medical diagnostics either for home testing (as in the case of home pregnancy tests), point-of-care testing, or laboratory use.

2.1.10. Development of an Imunochromatography Assay for Fish ISAV

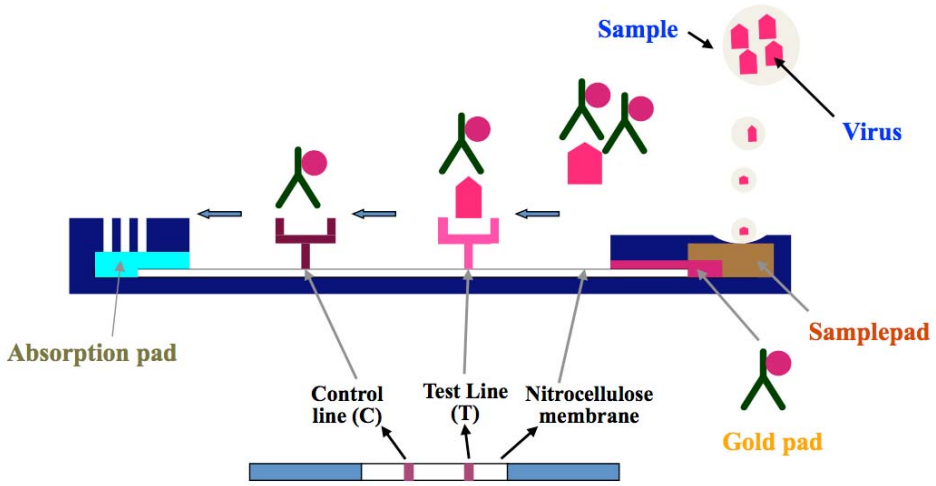

Figure 2.1.1. The principle behind the immunochromatography kit.

The technology is based on a series of capillary beds (sample pad, gold pad, and absorption pad) made of pieces of porous paper that can transport fluid spontaneously (Adams and Thompson, 2011). Usually, two monoclonal antibodies (mAbs) are applied, one is lined up on the nitrocellulose paper and the other is in the gold pad attached by gold particles. When a sample is poured into the hole on the sample pads, the pad acts as a sponge and once soaked, the fluid migrates to the gold pad in which mAbs are stored and reacts with a corresponding antigen. The antigen and antibody mixture is flows through the porous structure and reach to the mAbs on the nitrocellulose paper. The mAbs capture the mixture of antigen-mAbs attached to gold particles. As more fluid pass, the first stripe

where monoclonal antibodies are lined up, golden particles accumulate and the strip area changes color. If there is no reaction with the captured mAbs, the fluid will reach the second strip lined with capture polyclonal antibody which will react with the monoclonal antibody attached to gold particles but did not react with the target. The second stripe will indicate whether or the device is working fine or not. After passing these reaction zones, the fluid enters the final porous material, the absorption pad, which simply acts as a waste container. The principle behind this technology is shown in Figure 2.1.1 while Figure 2.1.2 shows an example of this test.

Figure 2.1.2. An immunochromatography kit showing negative, positive and invalid results.

2.2. Diagnosis – PCR Detection

Takashi Aoki and Jun-ichi Hikima

2.2.1. Synopsis

A quick, reliable, and efficient diagnostic method is indispensable to fish farm management and will help in identifying proper therapeutic measures and preventing the spread of diseases. The recent advances in genetic engineering and molecular biology made it possible to detect a specific gene in the target pathogen. The ability of polymerase chain reaction (PCR) to accurately detect viral or bacterial genes in a relatively shorter period compared to other diagnostic methods, made it one of the most widely used diagnostic tool for detecting viral and bacterial pathogens in fish. In this section, the basic principles of PCR assay, the bacteria and viruses in fish to which a PCR platform for detection and diagnosis was developed, and the target genes in these pathogens that were used for detection, will be introduced.

2.2.2. Introduction

As the number of fish and shellfish species for aquaculture increases to augment the need for cheaper food sources, the development of aquaculture technologies and diversification of demand for them also increases. With this increase however, comes the emergence of new diseases and disease-causing microbes associated with these new cultured species. Diseases caused by new bacterial and viral pathogens are causing huge damages and to address this concern, proper prophylactic and therapeutic methods are necessary. More importantly, rapid and accurate diagnostic tools to detect specific pathogens need to be developed.

Historically, morphological observation, biochemical and immunological (using antiserum) methods have been used for general identification and diagnosis of major pathogenic bacteria and virus including fish pathogens. It has also been done by comparison of nucleic acids, components and substances produced from the pathogens. However, since all of these methods are complicated and requires considerable time for detection, they are not suitable for use in aquaculture farms.

Recently, through the advances of technology in genetic engineering and molecular biology, it is possible to detect a specific gene in the target pathogen. The morphological and biochemical features of pathogens are basically determined by the genome (genes) derived from pathogens. By detecting a unique gene for a target pathogen, Polymerase Chain Reaction (PCR) diagnosis method, which is able to perform the rapid identification and precise classification, has been developed.

In this sub-section, the PCR method, currently one of the most popular diagnostic methods to identify fish pathogenic bacteria and viruses will be introduced.

2.2.3. The Basic Principles of PCR

PCR technology was developed by Dr. Karrie B. Mullis in 1987 using a thermophilic bacterial DNA polymerase (Taq DNA polymerase) that works efficiently even at high temperatures. Since the development of the PCR technique, research in the field of molecular biology improved in leaps and bounds. Now, it is one of the most widely used techniques not just in molecular biology, but also in most other scientific fields for its many advantages.

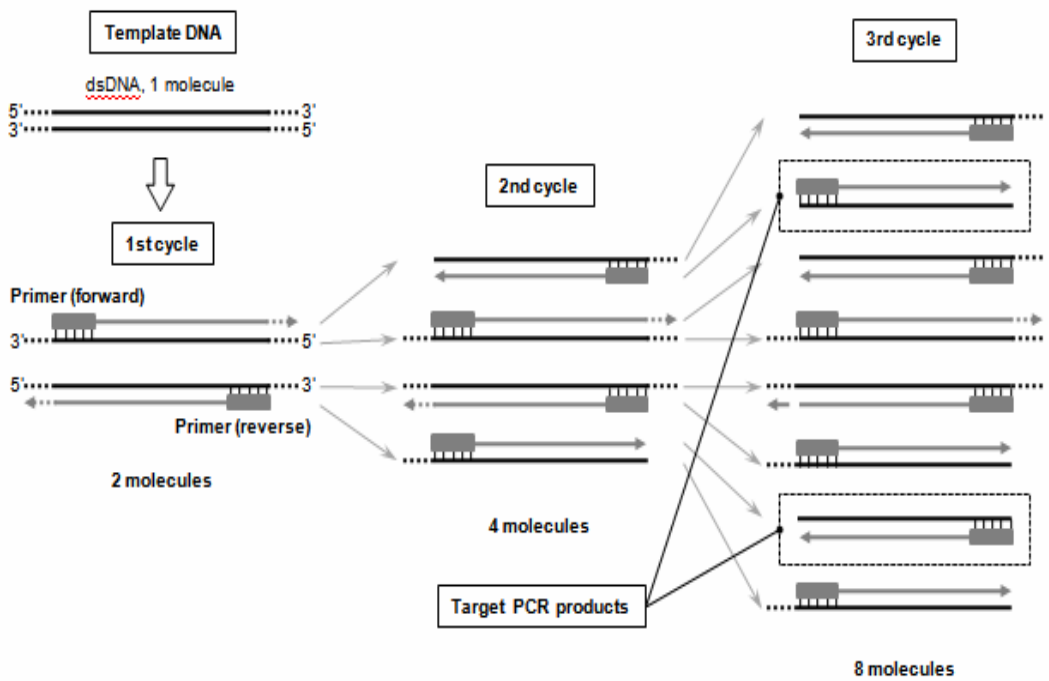

Figure 2.2.1. Schematic drawing of the basic principle of PCR technology. Grey box indicates primers, and grey arrow is the DNA fragment synthesized with Taq DNA polymerase, which synthesizes DNA from 5' to 3' direction. Target PCR products (specific DNA fragments) can be obtained in the 3rd cycle.

PCR functions to repeat only certain regions of DNA replication and amplification reaction and amplify DNA fragments with the same nucleotide exponentially, and generate large amounts of specific DNA fragments within a short time. PCR reaction is composed of the following steps: 1) dissociation (denaturing) of double-stranded DNA (dsDNA) (complementary DNA as template) by high temperature; 2) annealing of primers to the denatured template DNA (hybridizing the primer and the single-stranded DNA); and 3) synthesis of the complementary strand DNA with Taq DNA polymerase. By repeating these steps about 25 to 35 cycles, the certain area (*e.g.*, targeted gene) specified by two primers (*i.e.*, forward and reverse primers) is capable of amplifying 2^{25} to 2^{35} fold of the target DNA fragments (Fig. 2.2.1). Primers are 18- to 30-mer oligonucleotide DNA fragments, which specifically bind to the complementary strand of the target dsDNA by hydrogen bonds in each PCR cycle.

2.2.4. Diagnosis of Fish Bacterial Pathogens by PCR

PCR is a simple, rapid and more accurate, compared to other diagnostic techniques, method to identify specific genes of pathogenic bacteria and virus and is therefore widely used as a detection method for various fish and shellfish pathogenic bacteria and viruses. To date, the following bacterial pathogens in fish have been diagnosed by PCR using specific primer sets: *Edwardsiella ictaluri* and *Ed. tarda* (edwardsiellosis), *Tenacibaculum maritimum* (flexibacteriosis), *Flavobacterium columnare* (columnaris disease), *Renibacterium salmoninarum* (Bacterial kidney disease: BKD), pathogen of Bacterial hemolytic jaundice (unidentified species), *Pseudomonas anguilliseptica* (red spot disease), *Aeromonas salmonicida* (furunculosis), *Nocardia seriolae* (nocardiosis), *V. anguillarum*, *V. trachuri*, *V. vulunificu* (vibriosis), *Mycobacterium marinum* (mycobacteriosis), *Photobacterium damselae* subsp. *piscicida* (pseudotuberculosis), *F. psychrophilum* (cold water disease), *Y. ruckeri* (enteric redmouth disease), *Lactococcus garvieae*、*Streptococcus iniae*、*S. dysgalactiae*、*S. agalactiae*、*S. parauberis*、*S. difficilis* (streptococcosis) (Table 2.2.1).

Meanwhile, fish viruses detected by PCR method include, koi herpes virus (KHV), *Oncorhynchus masou* virus (OMV), channel catfish virus (CCV), red seabream iridovirus (RSIV), lymphocystis disease virus (LCDV), epizootic haematopoietic necrosis virus (EHNV), infectious pancreatic necrosis virus (IPNV), infectious salmon anemia virus (ISAV), infectious haematopoietic necrosis virus (IHNV), viral haemorrhagic septicemia virus (VHSV), spring viraemia of carp virus (SVCV), viral nervous necrosis virus (VNNV), salmonid alphavirus (SAV), grass carp reovirus (GCRV), European catfish virus (EGV), and hirame rhabdovirus (HRV) (Table 2.2.1).

2.2.5. Target Genes for PCR-Based Diagnosis

16s and 23S rRNA (or rDNA) genes and related genes (including ISR region or ITS genes located between 16S and 23S rRNA genes) has been frequently used as target genes to detect fish pathogens by PCR method. The following genes are also used for PCR diagnosis as the target gene: major outer membrane protein gene *p57* derived from *R. salmoninarum* (Brown *et al*., 1994; McIntosh *et al*., 1996; Miriam *et al*., 1997), surface array protein gene *vapA* in *A. salmonicida* (Gustafson *et al*., 1993), glutamine synthetase gene *glnA* in *Y. ruckeri* (Keeling *et al*., 2012), dihydropteroate synthase gene in *L.*

garvieae (Aoki *et al*., 2000), Lactate oxidase gene *lctO* in *S. iniae* (Hussein and Hatai, 2006; Mata *et al*., 2004a) (Table 1). In other pathogenic bacteria, such as the causative agent for vibriosis, there have been many evidences using genes related to virulence as the target gene for PCR detection namely: Hemolysin gene (Hirono *et al*., 1996), *rpoS* gene (Kim *et al*., 2008), *empA* gene (Xiao *et al*., 2009), *toxR* gene (Crisafi *et al*., 2011), cytotoxin-hemolysin gene (Coleman *et al*., 1996; Hill *et al*., 1991) (Table 2.2.1). Furthermore, a certain region in species-specific plasmid pZP1 (Aoki *et al*., 1997) and the species-specific sequences in the chromosomal DNA obtained by RAPD (Random Amplification of Polymorphic DNA)-PCR method or random cloning method (Aoki and Hirono, 1995; Aoki *et al*., 1995; Argenton *et al*., 1996; Iwamoto *et al*., 1995; Miyata *et al*., 1996) are also useful for PCR diagnosis.

Target genes for PCR detection of fish viruses on the other hand are: thymidine kinase and terminase for KHV (Bercovier *et al*., 2005; Yuasa *et al*., 2012); major capsid protein for OMV and LCDV (Aso *et al*., 2001; Kitamura *et al*., 2006; Cano *et al*., 2007; Palmer *et al*., 2012); ORF 8 for CCV (Gray *et al*., 1999); reductase, ATPase and DNA polymerase for RSIV (Ohima *et al*., 1996; 1998; Kurita *et al*., 1998); DNA polymerase for EHNV and ECV (Holopainen *et al*., 2011); VP1, VP3 and VP4 for IPNV (Willisms *et al*., 1999; Rodriguez *et al*., 2001; Orpetveit *et al*., 2010; Bowers *et al*., 2008); segment 8 for ISAV (Devold *et al*., 2000); the nucleoprotein or glycoprotein genes for IHNV, VSHV, SVCV and HRV (Williams *et al*., 1999; Arakawa *et al*., 1990; Bruchhof *et al*., 1995; Miller *et al*., 1998; Liu *et al*., 2008; Lopez-Vazquez *et al*., 2006; Chico *et al*., 2000; Koutna *et al*., 2003; Sun *et al*., 2010); coat protein for VNNV (Dalla *et al*., 2000); NSP1 for SAV (Zhang *et al*., 2010); and segment 10 for GCRV (Hodneland and Endresen, 2006) (Table 2.2.1).

Because of its effectiveness, PCR has revolutionized modern pathogen-diagnostics and it has been developed to detect a wide variety of bacterial and viral pathogen. It is easy to use, rapid and accurate making it a very excellent diagnostic method for fish pathogens.

2.2.6. Conclusion

Farm-level diagnostic tools that are cost-effective, easy to use, and allows for rapid detection of well known pathogens will greatly improve aquaculture outputs. The PCR-based diagnostic method embodies these characteristics very well and has proven to be reliable if not more reliable than traditional methods for disease detection. Although this method has evolved and developed through the years, the need to optimize detection, sensitivity, and accuracy is required to expand its utility and versatility. The development according to the intended use and purpose of further diagnosis will be anticipated.

Diseases	**Causative agent**	**Target genes**	**Primer sequences（F: forward / R: reverse）**	**Products（bp）**	**References**
Edwardsiellosis	*Edwardsiella ictaluri*	Region between IVS-IRS genes	F:5'TTAAAGTCGAGTTGGCTTAGGG3', R:5'TACGCTTTCCTCAGTGAGTGTC3'	2,000	William and Lawrence, 2010
	Ed. tarda	*Eta1*（Species-specific DNA fragments）	F:5'AGTTCAGCGCCCAGTCATA3', R:5'CGCCAGATCCGCTGCCCGT3'	580	Aoki and Hirono, 1995
Flexibacteriosis	*Tenacibaculum maritimum* (Former name: *Flexibacter maritimus*)	16S rRNA gene	F:5'AATGGCATCGTTTTAAA3', R:5'CGCTCCTACTTGCGTAG3'	1073	Toyamaet al., 1996
		16S rRNA gene	F:5'TGTAGCTTGCTACAGATGA3', R:5'AAATACCTACTCGTAGGTACG3'	400	Bader and Shotts, 1998; Cepeda et al., 2003
		16S rRNA gene	F:5'AATGGCATCGTTTTAAA3', R:5'CGCTCCTACTTGCGTAG3', F(nested):5'AGAGTTTGATCCTGGCTCAG3', R(nested):5'AAGGAGGTGATCCAGCCGCA3'	1088	Avendano-Herrera et al., 2004
Columnaris disease	*Flavobacterium columnare*	16S rRNA gene	F:5'GCCCAGAGAAATTTGGAT3', R:5'TGCGATTACTAGCGAATCC3'	1,193	Bader et al., 2003
		16S rRNA gene	F:5'CAGTGGTGAAATCTGGT3', R:5'GCTCCTACTTGCGTAGT3'	679	Darwish et al., 2004
		ISR region between 16S-23S rRNA genes	F:5'TGCGGCTGGATCACCTCCTTTCTAGAGACA3', R:5'TAATYRCTAAAGATGTTCTTTCTACTTGTTTG3'	450~550	Welker et al., 2005
Bacterial kidney disease (BKD)	*Renibacterium salmoninarum*	16S rRNA gene	F:5'TGGATACGACCTATCACCGCA3', R:5'GCAAGTACCCTCAACAACCACA3'	312	Magnússon et al., 1994
		*p57*major outer membrane protein gene	F:5'CAAGGTGAAGGGAATTCTTCCACT3', R:5'GACGGCAATGTCCGTTCCCGGTTT3'	501	Brown et al., 1994
		*p57*major outer membrane protein gene	F:5'GCGCGGATCCAAAATAAAAAAAATTTTAGCGCTG3', R:5'GCGCGGATCCTTGGCAGGACCATCTTTGT3'	376	McIntosh et al., 1996
		*p57*major outer membrane protein gene	F:5'CGCAGGAGGACCAGTTGCAG3', R:5'GGAGACTTGCGATGCGCCGA3'	349	Miriam et al., 1997
		*p57*major outer membrane protein gene	F:5'CGCAGGAGGACCAGTTGCAG3', R:5'TCCGTTCCCGGTTTGTCTCC3'	372	Miriam et al., 1997

		16S-S23 rDNA ITS gene	F:5'CCGTCCAAGTCACGAAAGTTGGTA3', R:5'ATCGCAGATTCCCACGTCCTTCTT3'	751	Grayson et al., 1999
		16S-S23 rDNA ITS gene	F:5'CCGTCCAAGTCACGAAAGTTGGTA3', R:5'GTGGGTACTGAGATGTTTCAGTTC3'	895	Grayson et al., 1999
Bacterial hemolytic jaundice	Unidentified	16S rDNA gene	F:5'AGCACTTATGTATAGGTGTA3', R:5'GTATAAAACGCCAAACATAT3'	387	Mitsui et al., 2004 (In Japanese)
Red spot disease	*Pseudomonas anguilliseptica*	16S rRNA gene	F:5'GACCTCGCCATTA3', R:5'CTCAGCAGTTTTGAAAG3'	439	Blanco et al., 2002
Furunculosis	*Aeromonas salmonicida*	*vapA* gene	F:5'GGCTGATCTCTTCATCCTCACCC3', R:5'CAGAGTGAAATCTACCAGCGGTGC3'	421	Gustafson et al., 1992, 1993
		16S rRNA gene	F:5'CGTTGGATATGGCTCTTCCT3', R:5'CTCAAAACGGCTGCGTACCA3'	423	O'Brien et al., 1994
	A. salmonicida subsp. *salmonicida*	Species-specific region in chromosome (RAPD products)	F:5'AGCCTCCACGCGCTCACAGC3', R:5'AAGAGGCCCCATAGTGTGGG3'	512	Miyata et al., 1996
Nocardiosis	*Nocardia seriolae*	16S rRNA gene	F:5'ACTCACAGCTCAACTGTGG3', R:5'ACCGACCACAAGGGGG3'	432	Miyoshi and Suzuki, 2003
Vibriosis	*Vibrio anguillarum*	Hemolysin gene	F:5'ACCGATGCCATCGCTCAAGA3', R:5'GGATATTGACCGAAGAGTCA3'	490	Hirono et al., 1996
		rpoS gene	F:5'AGACCAAGAGATCATGGATT3', R:5'AGTTGTTCGTATCTGGGATG3'	689	Kim et al., 2008
		empA gene	F:5'CAGGCTCGCAGTATTGTGC3', R:5'CGTCACCAGAATTCGCATC3'	439	Xiao et al., 2009
		toxR gene	F:5'ACACCACCAACGAGCCTGA3', R:5'TTGTCTCTTCGGGTTGCGA3'	93	Crisafi et al., 2011
Vibriosis	*V. anguillarum*	16S rRNA gene	F:5'CCACGCCGTAACGATGTCTA3', R:5'CCAGGCGGTCTACTTAACGCGT3'	81	Crisafi et al., 2011
	V. trachuri	Species-specific region in chromosome	F:5'TGCGCTGACGTGTCTGAATT3', R:5'TGACGAACAGTAGCGACGAA3'	417	Iwamoto et al., 1995
	V. vulunificu	Cytotoxin-hemolysin gene	F:5'CCGGCGGTACAGGTTGGCGC3', R:5'CGCCACCCACTTTCGGGCC3'	519	Hill et al., 1991
		23S rRNA gene	F:5'CCACTGGCATAAGCCAG3', R:5'CTACCCAATGTTCATAGAA3'	978	Arias et al., 1995
		Cytolysin-hemolysin gene	F:5'CGCCGCTCACTGGGGCAGTGGCTG3', R:5'GCGGGTGGTTCGGTTAACGGCTGG3'	1416	Coleman et al., 1996

Mycobacteriosis	*Mycobacterium spp., Mycobacterium marinum*	16S rRNA gene	F:5'GRGRTACTCGAGTGGCGAAC3', F:5'GGCCGGCTACCCGTCGT3'	208	Kox et al., 1995, 1997; Puttinaowarat et al., 2002
Pasteurellosis (Pseudotuberculosis)	*Photobacterium damselae* subsp. *piscicida* (Former name: *Pasteurella piscicida*)	Species-specific region in chromosome	F:5'GTAGCTCTTGTGGAGTAATGCT3', R:5'CATTCGTAGTGCTTACTGCCCA3'	629	Aoki et al., 1995
		DNA fragment from pZP1	F;5'GCCCCCATTCCAGTCACACA3', R:5'TCCCTAAGCACACCGACAGG3'	484	Aoki et al., 1997
		16S rRNA gene	F:5'CGAGCGGCAGCGACTTAACT3', R:5'GATTACCAGGGTATCTAATC3'	~750	Matsuoka et al., 1997 (In Japanese)
Cold water disease	*F. psychrophilum* (Former name: *Cytophaga psychrophila*)	16S rRNA gene	F:5'CGATCCTACTTGCGTAG3', R:5'GTTGGCATCAACACACT3'	1073	Toyama et al., 1994
		16S rRNA gene	F:5'GTTAGTTGGCATCAACAC3', R:5'TCGATCCTACTTGCGTAG3'		Urdaci et al., 1998
Enteric redmouth Disease (ERM)	*Yersinia ruckeri*	Unidentified gene (RAPD-PCR products)	F:5'TCACGAATCAGGCTGTTACC3', R:5'TTCTGCCTGTGCCAATGTTGG3'	512	Argenton et al., 1996
		16S rRNA gene	F:5'GCGAGGAGGAAGGGTTAAGTG3', R:5'GAAGGCACCAAGGCATCTCTG3'	575	Gibello et al., 1999
		glnA gene	F:5'TCCAGCACCAAATACGAAGG3', R:5'ACATGGCAGAACGCAGATC3', Probe:5'CGCGATCAAGGCGGTTACTTCCCGGTTCCCGATCGCG3'(Real-time PCR)	ND	Keeling et al., 2012
Streptococcosis	*Lactococcus garvieae*	16S rDNA gene	F:5'CATAACAATGAGAATCGC3', R:5'GCACCCTCGCGGGTTG3'	1,100	Zlotkin et al., 1998a; Hussein and Hatai, 2006
		Dihydropteroate synthase gene	F:5'CATTTTACGATGGCGCAG3', R:5'CGTCGTGTTGCTGCAACA3'	709	Aoki et al., 2000
	Streptococcus iniae	16S rDNA gene	F:5'CTAGAGTACACATGTACTNAAG3', R:5'GGATTTTCCACTCCCATTAC3'	300	Zlotkin et al., 1998b
		ITS region between 16S-23S rRNA genes	F:5'GGAAAGAGACGCAGTGTCAAAACAC3', R:5'CTTACCTTAGCCCCAGTCTAAGGAC3'	373	Berridge et al., 1998
		Lactate oxidase (*lctO*) gene	F:5'AAGGGGAAATCGCAAGTGCC3', R:5'ATATCTGATTGGGCCGTCTAA3'	870	Mata et al., 2004a; Hussein and Hatai, 2006

		ITS region between 16S-23S rRNA genes	F:5'GAAAATAGGAAAGAGACGCAGTGTC3', R:5'CCTTATTTCCAGTCTTTCGACCTTC3'	377	Zhou et al., 2011
		16S rDNA gene	F:5'CTAGAGTACACATGTACTIAAG3', R:5'GGATTTTCCACTCCCATTAC3'	300	Roach et al., 2006
	S. dysgalactiae	ITS region between 16S-23S rRNA genes	F:5'TGGAACACGTTAGGGTCG3', R:5'CTTTTACTAGTATATCTTAACTA3'	270	Forsman et al., 1997
	S. dysgalactiae subsp. *dysgalactiae*	ITS region between 16S-23S rRNA genes	F:5'TGGAACACGTTAGGGTCG3', R:5'CTTAACTAGAAAAACTCTTGATTATTC3'	259	Hassan et al., 2003; Hussein and Hatai, 2006
	S. agalactiae	ITS region between 16S-23S rRNA genes	F:5'GGAAACCTGCCATTGCG3', R:5'TAACTTAACCTTATTAACCTAG3'	280	Forsman et al., 1997
	S. parauberis	23S rRNA gene	F:5'TTTCGTCTGAGGCAATGTTG3', R:5'GCTTCATATATCGCTATACT3'	718	Mata et al., 2004b
	S. difficilis	ITS region between 16S-23S rRNA genes	F:5'AGGAAACCTGCCATTTGCG3', R:5'CAATCTATTTCTAGATCGTGG3'	192	Mata et al., 2004b

Table 2.2.1. Fish pathogenic bacteria detected by PCR methods

Glossary

PCR: polymerase chain reaction,

dsDNA: Double-stranded deoxyribonucleotide,

RAPD: Random Amplification of Polymorphic DNA,

KHV: Koi herpes virus,

OMV: *Oncorhynchus masou* virus,

CCV: Channel catfish virus,

RSIV: Red seabream iridovirus,

LCDV: Lymphocystis disease virus,

EHNV: Epizootic haematopoietic necrosis virus,

IPNV: Infectious pancreatic necrosis virus,

ISAV: Infectious salmon anemia virus,

IHNV: Infectious haematopoietic necrosis virus,

VHSV: Viral haemorrhagic septicemia virus,

SVCV: Spring viraemia of carp virus,

VNNV: Viral nervous necrosis virus,

SAV: Salmonid alphavirus,

GCRV: Grass carp reovirus,

EGV: European catfish virus,

HRV: Hirame rhabdovirus

2.3. Loop Mediated Isothermal Amplification (LAMP) Method

Masahiro Sakai

2.3.1. Synopsis

The LAMP (loop mediated isothermal amplification) can amplify nucleic acids with high specificity, sensitivity and rapidity under isothermal conditions. The LAMP reaction employs a DNA polymerase and a set of four specific primers that recognize a total of six distinct sequences of the target DNA. In aquaculture, this technique has already been applied for detection of several fish and shrimp pathogens such as KHV, SVCV, IHNV, WSSV, *Edwardsiella tarda*, *Vibrio nigripulchritudo*, YHV, *Nucleospora salmonis* etc. According to these reports, the sensitivity of LAMP is almost the same as PCR and suggesting that this technique can be used for diagnosis of these diseases. Furthermore, the real-time LAMP method has been recently developed for quantitative detection of pathogens.

2.3.2. Principle of LAMP

Loop-mediated isothermal amplification is a sensitive strand displacement technique developed by Notomi *et al.* (2000). This method amplifies target DNA from a few copies to 10^9 copies in less than an hour under isothermal conditions. Briefly, four specific primers are designed 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 using *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.

2.3.3. Design of Primers

Designing primers for LAMP is a complex procedure compared with the PCR. The LAMP reaction requires four primers. The primers required are one pair each of inner-primers and shorter outer-primers (Figure 2.3.1). Although the design of each primer is very complex, it can be developed using Primer Explorer version 3 software (http://primerexplorer.jp/lamp3.0.0/).

2.3.4. Requirements for LAMP Reaction

The LAMP reaction is performed by the *Bst* polymerase along with dNTP's and reaction buffer. The reaction is carried out at 60-65°C for 40 min to 1 hour and terminated at 80°C for 2 min. The main advantage of the technique is that it does not need a thermocycler. As the amplification is done in an isothermal condition, a water bath or heating block is sufficient to maintain the required temperature.

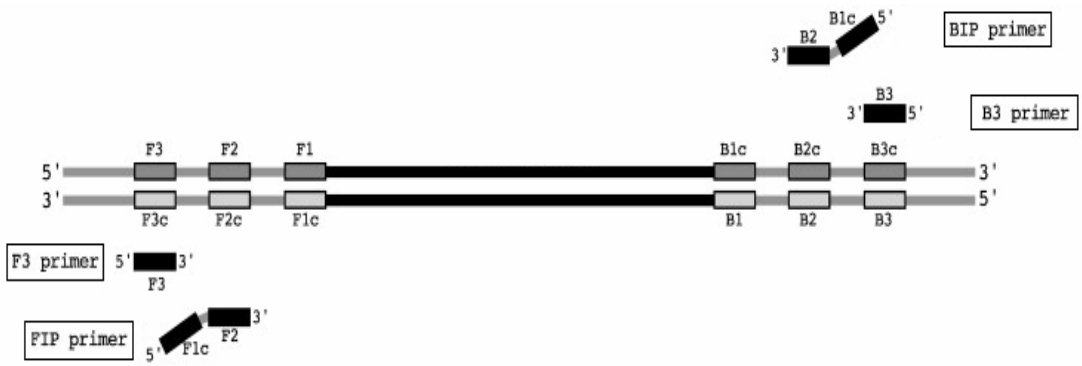

Figure 2.3.1. (A) Schematic diagram of two-inner (FIP, BIP) and -outer (F3, B3) primers for LAMP. This diagram was adapted from Eiken Chemical Co. Ltd (This Figure was modified and cited from Aquaculture, Vol. 288, p27-31 (2009).

2.3.5. Visualization of Amplified Products

The amplified products by LAMP are commonly visualized by agarose gel

electrophoresis stained with ethidium bromide. As the LAMP reaction produces products of various lengths of stem loop structures, the gel will show a smear and bands at the base of the gel (Figure 2.3.2). Furthermore, the large amount of product amplified by LAMP can be visualized on a UV-transilluminator by incorporating intercalating agents, such as SYBR Green I, directly into the LAMP-amplified tubes (Notomi et al, 2000). For alternative method, these products are assessed by the amount of white precipitate formed from magnesium pyrophosphate (Mori et al, 2001).

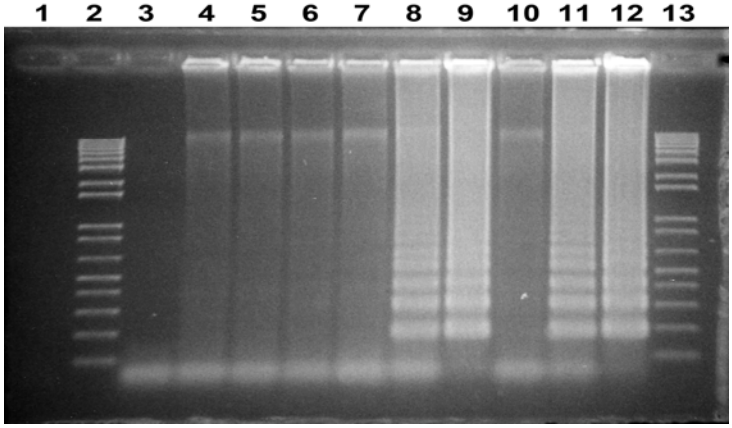

Figure 2.3.2. Determination of LAMP conditions. Effect of temperature and time on amount of LAMP product. Temperature: Lanes 2 and 13: molecular size marker (φ/X174/Hinc II digest), lane 3: blank, lanes 4–6: 60°C, lanes 7–9: 63°C, lanes 10–12: 65°C. Time: Lanes 4, 7 and 10: 30 min, lanes 5, 8 and 11: 45 min, lanes 6, 9 and 12: 60 min. Products were electrophoresed on a 2% agarose gels and stained with ethidium bromide (This Figure was cited from Aquaculture, Vol. 288, p27-31 (2009)).

2.3.6. Application of LAMP for Diagnosis of Fish Pathogens

Since the 2000s, LAMP method has been widely used for detecting human pathogens, because of its simplicity, rapidity, high efficiency, and outstanding specificity. In fisheries sciences, the first use of LAMP for detection of an aquaculture pathogen was reported by our group (Savan et al, 2004). Until now, more than 50 articles covering detection of virus, bacteria and parasitic pathogen have been reported.

2.3.7. Bacterial Pathogens

Many reports on LAMP mediated diagnostic methods have been developed for bacterial pathogens associated with fish and shrimp. The first use of LAMP for detection of aquaculture pathogen was reported for Edwardsiellosis (Savan et al, 2004). LAMP primers were designed by targeting the hemolysin gene of *Edwardsiella tarda*. The specificity of LAMP was tested for 5 different *E. tarda* strains and non-specific amplification was not seen in other bacteria. The optimum amplification was determined to be at 65°C for 45 min. *E. tarda* could be detected from 10 CFU and 10^3 CFU by LAMP and PCR, respectively. LAMP method has also been applied for the detection of Nocardiosis (Itano et al, 2006). In Nocardiosis, the detection limits of LAMP and PCR were 10^3 CFU and 10^4 CFU, respectively. Compared to PCR, a ten fold higher sensitivity

is observed using LAMP. Furthermore, LAMP detection was superior to PCR, when spleen DNA extracted from infected fish was used as template. In shrimp pathogen, the detection of *Vibrio nigripulchritudo* was established by Fall et al. (Fall et al, 2008). Reaction time and temperature were optimized for 60 min at 63°C, respectively and the detection limit of this bacterium by LAMP was 10^2 CFU. The application of LAMP method to diagnose other bacterial fish and shrimp pathogens is shown in Table 2.3.1.

2.3.8. Detection of Viruses

2.3.8.1. DNA Viruses

In the detection of fish viral pathogens, LAMP was first applied in koi herpesvirus (KHV). A set of four primers were designed based on the sequence of the thymidine kinases gene of KHV (Gunimaladevi et al, 2004). The time and temperature conditions for detection of KHV were determined for 60 min at 65ºC. The detection limit using LAMP was found to be similar to that of PCR. Detection of WSSV infecting kuruma shrimp, *Marsupenaeus japonicus*, was reported by Kono *et al*. (Kono et al, 2004). The detection limit of the viral DNA template was 10 fg level, while nested PCR mediated detection limit was 100 fg level. The study concluded that detection by LAMP was superior to PCR since it was faster and more sensitive. Yoshino *et al.* (Yoshino et al, 2006) reported the diagnosis method of KHV using additional sets of loop primers. Additionally, LAMP has also been used to detect red sea-bream iridovirus (Caipang et al, 2004) and infectious hypodermal and hematopoietic necrosis virus (IHHNV) (Sun et al, 2006).

Pathogen	**Method**
Bacteria	
Edwardsiella tarda (Savan et al. 2004)	LAMP
Edwardsiella ictaluri (Yeh et al. 2005)	LAMP
Flavobacterium columnare (Yeh et al. 2006)	LAMP
Nocardia seriolae (Itano et al., 2006)	LAMP
Yersinia ruckeri (Saleh et al., 2008)	LAMP
Flavobacterium psychrophilum (Fugiwara-Nagata, Eguchi, 2009)	qLAMP
Renibacterium salmoninarum (Gahlawat et al. 2009)	LAMP
Mycobacterium sp. (Ponpompisit et al., 2009)	LAMP
Vibrio anguillarum（Hongwei et al., 2010）	LAMP
Francisella piscicida (Caipang et al., 2010)	LAMP
Vibrio nigripulchritudo (Fall et al., 2011)	qLAMP
Streptococcus iniae (Han et al., 2011)	LAMP
Viruses	
KHV (Gunimaladevi et al., 2004)	LAMP
WSSV (Kono et al., 2004)	LAMP
IHNV (Gunimaladevi et al., 2005)	RT-LAMP
YHV (Mekata et al., 2006)	RT-LAMP
SVCV (Shivappa et al., 2008)	RT-LAMP
VHSV (Soliman and El-Matbouli 2006)	RT-LAMP
IHHNV (Sudhakaran et al., 2008)	qLAMP

WSSV (Mekata et al., 2009)	qLAMP
YHV (Mekata et al., 2009)	qRT-LAMP
IPNV (Soliman at al., 2009)	RT-LAMP
Iridovirus (Caipang et al., 2004)	LAMP
NNV (Sung and Lu 2009)	RT-LAMP
Parasites	
Tetracapsuloides bryosalmonae (El-Matbouli and Soliman 2005)	LAMP
Myxobolus cerebralis (El-Matbouli and Soliman 2005)	LAMP
Nucleospora salmonis (Sakai et al., 2009)	LAMP
Clonorchis sinensis (Cai et al., 2010)	LAMP

Table 2.3.1. Fish and shrimp pathogens detected using LAMP

2.3.8.2. RNA Viruses

For detecting RNA viruses, the cDNA from the virus RNA must be synthesized by reverse transcription. After the development of the LAMP method, an extended application of RT-LAMP has been developed (Notomi et al, 2000). In fish, RT-LAMP was first reported for IHNV (Gunimaladevi et al, 2005). An RT-LAMP protocol for detection of IHNV was developed targeting the G-protein of the virus. A comparative analysis of RT-LAMP, LAMP and nested PCR was conducted. LAMP and nested PCR require an additional 30-40 min as cDNA should be synthesized first. However, RT-LAMP can directly use RNA as template, where the cDNA synthesis and target gene amplification is carried out in a single tube. In this study, LAMP was 10-fold more sensitive than nested PCR. Although real-time PCR is a superior method, RT-LAMP might be a good alternative as the former can be expensive as a routine diagnostic tool. The use of RT-LAMP has also been reported in the detection of viral hemorrhagic septicemia virus (VHS) of salmonid fish (Salivan and El-Matbouli 2006), yellow head virus in shrimp (Mekata et al, 2006), spring viremia of carp (SVC) (Shivappa et al, 2008) and infectious salmon anemia virus (ISAV) (Sakai, personal communication).

2.3.9. Parasitic Infections

LAMP method has also been applied for detecting fish parasitic diseases. A myxozoan spore, *Tetracapsuloides bryosalmonae*, is the causative agent of proliferative kidney disease (PKD). Et-Matbouli and Soliman (El-Matbouli and Soliman, 2005a) used LAMP for rapid diagnosis of (PKD) affected rainbow trout. Furthermore, a comparison of PKD-LAMP to PCR has been evaluated in this study. Four sets of primers along with loop primers were designed targeting SSU-rDNA of *T. bryosalmonae*. The loop primers were used for the acceleration of LAMP reaction. The PKD-LAMP was found to be 100-fold more sensitive and a low amount of DNA sample as template could also be amplified in 1 h. In addition, Et-Matbouli and Soliman (El-Matbouli and Soliman 2005b) have also reported a detection method based on LAMP for *Myxobolus cerebralis*, which is a causative agent of whirling disease.

2.3.10. Quantitative LAMP Method

A quantitative real-time LAMP method has been reported (Mori, et al., 2001). This method produces large amounts of the target DNA as well as an insoluble by-product, magnesium pyrophosphate, during the reaction making it possible to perform a real-time measurement of turbidity using an inexpensive photometer. Sudhakaran et al. (2008) reported the real-time LAMP assay to detect IHHNV in shrimp. The real-time LAMP method for IHHNV is simple and rapid with specific amplification within 60 min at 63ºC. The sensitivity analysis revealed this method is capable of detecting as few as 10^2–10^3 copies/μL. This method was also reported in the detection of WSSV (Mekata et al, 2009a) and YHV (Mekata et al, 2009b).

2.3.11. Conclusion

This review describes the application of LAMP method for detection of fish and shellfish pathogens. Various studies cited in this review have convincingly demonstrated that LAMP is a superior diagnostic tool compared to other methods. This method can be widely applied in clinical diagnostics, environmental monitoring and food safety in aquatic sciences.

Glossary

LAMP: Loop Mediated Isothermal Amplification,

dNTP: Mixture of dATP (deoxyadenosine triphospate) + dCTP (deoxycytidine triphospate) + dGTP (deoxyganosine triphospate) + dTTP (deoxytymidine triphospate)

3. SELECTION AND ESTABLISHMENT OF DISEASE-RESISTANT FISH

3.1. Development of Disease-Resistant Fish Using Marker-Assisted Selection

Takashi Sakamoto, Akiyuki Ozaki and Nobuaki Okamoto

3.1.1. Synopsis

In aquaculture, one way to prevent fish diseases is to develop disease-resistant strains of fish through the use of marker-assisted selection (MAS). MAS requires an understanding of the linkage between quantitative trait loci (QTL) of a target trait and DNA markers. Presently, detection of disease-resistant phenotypes requires artificial challenge tests, which are labor intensive and expensive. However, such tests are no longer needed once the linkage between disease resistance traits and DNA markers is known. So far, MAS has been used to develop Japanese flounder resistant to lymphocystis disease (LD) and Atlantic salmon resistant to infectious pancreatic necrosis (IPN).

3.1.2. Introduction

The majority of species and strains reared globally for aquaculture are relatively unimproved for commercially important traits. Presently, cultured and wild fish species