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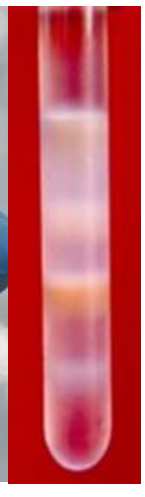
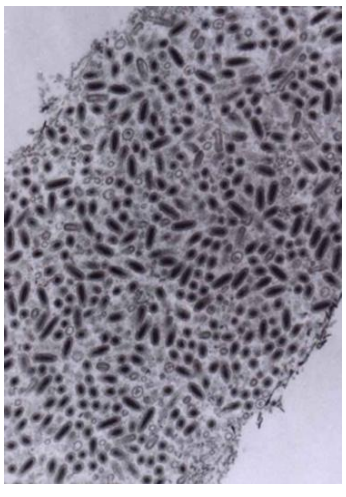
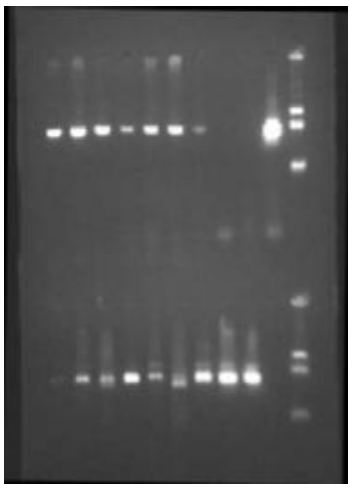
ICAR - CENTRAL INSTITUTE OF BRACKISHWATER AQUACULTURE

Molecular Diagnosis of Shrimp Diseases

- A Training Manual

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Preface

The Indian aquaculture production has considerably improved during the last five years, especially after the introduction of Pacific white shrimp to a new level of about 0.35 million tonnes of farmed shrimp. Availability of imported specific pathogen free (SPF) broodstock provided the much needed impetus to India's brackishwater aquaculture sector. However, progressive intensification has exacerbated the epizootics and disease issues are affecting productions. The white spot virus disease (WSD) caused by white spot syndrome virus (WSSV), results in and is continuing to pose a serious challenge with significant crop losses. As there are no therapeutic options available for the viral disease, and the only option available was to detect and avoid the pathogen entry into culture systems the hatcheries and grow-out farms, using smart diagnostic tools based on DNA technology such as polymerase chain reaction (PCR), with very high detection efficiency of few viruses in a short span of 3-4 hours. Until 2008, majority of the farmers screenedthe shrimp seed for WSSV in commercial PCR laboratories prior to stocking, and the practice helped to control the introduction of WSSV in the rearing systems and managed successful tiger shrimp *Peaneus monodon* crops.

However, after the introduction of the exotic specific pathogen free (SPF) Pacific white shrimp, *Penaeus vannamei* in the year 2009, farmers gave away the practice of PCR screening of shrimp seed, possibly due to wrong assumption of farmers that the seed originated from SPF stock, and PCR testing was not required. WSSV related crop losses along with new emerging disease situations such as running mortality syndrome (RMS), white faeces syndrome (WFS), stunted growth, white muscle syndrome (WMS) and the latest, shrimp hepatopancreatic microsporidosis caused by *Enterocytozoon hepatopenaei* (EHP) has therefore necessitated the screening of seed testing using smart DNA based diagnostic tools such as PCR before stocking. The CAA and CIBA have been emphasizing the use of only PCR tested shrimp seed for stocking.

In this backdrop, CIBA in association with CAA and RGCA under MPEDA is organising "Capacity building and ring test / harmonization of PCR diagnosis of aquatic animal diseases" programme for the benefit of the aquaculture sector in the country. This exercise includes three phases, (i) enrolment of laboratories / hatcheries with PCR labs and capacity building / training of technicians from the participating laboratories for a period of three days, (ii) inspection of the participating PCR laboratories, and (iii) PCR ring test. One of the purposes of ring test, also known as inter laboratory performance is to assure the farmers or regulatory officials that the results provided are accurate and specific, so that farmers could make the seed selection based on the results of PCR test, conducted at the private PCR labs approved after the ring test. Since the purpose of the ring test is not only to determine proficiency, but to help improve performance, feedback is also provided to those laboratories experiencing problems with the analysis, so that they get an opportunity for improvement to provide a quality service to the farmer.

The first ever PCR ring test was conducted in India at ICAR-CIBA, Chennai in the year 2005 under a regional project titled *"Application of PCR for improved shrimp health management in the Asian region"*with key partners including ACIAR, CSIRO and AusVet Services in Australia; MPEDA, CIBA and College of Fisheries, Mangalore in India; Mahidol University, BIOTEC and FAO-NACA in Thailand; Ministry of Marine Affairs and Fisheries in Indonesia. The second PCR ring testing exercise was conducted in the year 2009 by CIBA and MPEDA.

I welcome and record my appreciation of all these laboratories that have voluntarily come forward to participate in this ring test programme. These laboratories would play a major role in providing disease free seed to the aquafarmers in the country. This manual provides comprehensive information comprising protocols for shrimp sampling, nucleic acid extraction, performing PCR, electrophoresis to reporting the PCR results. A section on trouble shooting PCR is also included. This has been possible with dedicated scientific work of over 25 years of aquatic animal health related research at CIBA, which has the capacity for diagnosing all the OIE listed diseases of shrimp. Incidentally, CIBA is recognized as a National referral laboratory from brackishwater aquatic animal diseases by the Ministry of Agriculture and Farmers Welfare,

Govt. of India. This has been complemented by RGCA in terms of use of commercial diagnostic kits for detection of WSSV and their exposure in screening the samples for diseases. The efforts of scientists in putting up this comprehensive PCR manual in time would be highly beneficial to the PCR diagnostic laboratories serving the aquaculture sector, and also scientific and technical personnel engaged in aquatic animal disease surveillance work. I hope this effort would contribute significantly to prevention of diseases and the sustainability of aquaculture sector in the country.

K.K. Vijayan

Content

Exotics, Quarantine and Biosecurityfor Aquaculture Health Management

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Health maintenance in aquaculture is one of the most important aspects of aquaculture development and management. Realization of the importance of keeping pathogens away from rearing systems is of paramount importance for aquatic animal health management in aquaculture. The purpose of this article is to emphasize the importance of biosecurity and quarantine measures of aquaculture in minimizing risks of disease and maximizing aquaculture productions.

Introduction of exotic species, import risk analysis and quarantine

Aquatic animals are widely translocated across countries for enhancing aquaculture productions and species diversification. Introduction of exotic species has both benefits and adverse impact. The exotics may be introduced with a view to enhancing growth, genetic up-gradation of local stocks, as biological control agents, disease resistance and for ornamental purpose. The introduced species may escape into natural waters and establish in the wild. The adverse effect associated with exotics can be divided in to three main categories i.e. ecological, genetic and health. Ecological impacts may be due to habitat alteration, competition and predation. The genetic impact may result in the loss of native germplasm and genetic diversity, which has evolved through centuries to adapt to our conditions. For the economic, social and trade development, movement of live aquatic organisms across national boundaries is necessary even though such activities may lead to the introduction of new pathogens and pose risks to the importing country. Convention on Biodiversity (CBD) and the Biodiversity Act of India have to be also borne in mind while importing live aquatic organisms. Responsible fisheries emphasizes the need to minimize the risk of disease transfer and adverse effects on wild and cultured stocks associated with the introduction of non-native species and transport of eggs, larvae, brood stock and other live organisms. Presently ten fish, nine crustacean, seven molluscan and two amphibian diseases have been listed by the World Organization for Animal Health (OIE, 2016). In molluscs, parasitic diseases are important, while in fish and crustaceans, viral diseases are cause of concern. Whether a listed disease is due to a virus, fungus, bacterium or a parasite, the occurrence of the disease may adversely affect international trade among trading partners that have, or do not have, the listed disease.

As the movement of live aquatic animals involves certain degree of risk to the importing country, an import risk analysis (IRA) to assess the possible risk associated with the import is imperative. The main components of IRA are hazard identification, risk assessment, risk management and risk communication. Though various countries use different methods, they should be science-based and transparent with detailed documentation. The importing country has an obligation to ensure that IRA is scientifically sound, adequately documented and critically evaluated as per international obligations. The claims about its own aquatic animal health status should be accurate and based on scientific data and rigorous surveillance as demanded by the exporting country. The exporting country should also ensure that the information provided on its health status is accurate and based on international standards. It also has an obligation to report any significant change in the health status to all trading partners and international conventions. In India, *Penaeus vannamei*, a non-native species of shrimp, has been introduced to brackishwater aquaculture system as an alternative to *P. monodon*, culture, recently. The introduction was mainly driven by the negative impact of white spot disease (WSD) caused by white spot syndrome virus (WSSV) on the sustainability of *P. monodon* culture. The introduction of this exotic species was facilitated by

the Ministry of Agriculture, Govt of India, Coastal Aquaculture Authority and ICAR-CIBA and NBFGR. ICAR-CIBA and NBFGR carried out import risk analysis prior to the introduction of the species in the country and recommended for establishment and maintenance of quarantine facility by Government.

The strategy of the aquatic quarantine and health management system, primarily involves the protection of a country's aquatic biodiversity from exotic organisms, pathogens and containment of the diseases. The guiding principles in establishing a quarantine policy for responsible movement of aquatic organism and products have been formulated by consensus among various countries. According to FAO (2006), *"Quarantine means maintaining a group of aquatic animals in isolation with no direct or indirect contact with other aquatic animals, in order to undergo observation for a specified length of time and, if appropriate, testing and treatment, including proper treatment of the effluent waters.*" Detailed aquatic animal quarantine guidelines are provided in Asia Regional Technical Guidelines on Health Management for the Responsible Movement of Live Aquatic Animals and the Beijing Consensus and Implementation Strategy (FAO/NACA, 2000). For the success in formulating and implementing aquatic quarantine, health management and certification system, it is necessary to identify pathogens of concern. Viruses such as white spot virus and bacteria such as vibrios are of great concern. International Aquatic Animal Health code requires that all the member countries make available through OIE whatever information is necessary to minimize the spread of important pathogens and their etiological agents to assist in achieving better worldwide control of these diseases. The OIE has two lists of Diseases of Aquatic animals i.e. diseases notifiable to OIE and other significant diseases. These diseases, especially the notifiable ones, are of significance in international trade. At present, the OIE notifiable and other significant diseases are only considered for quarantine purposes. But there are other diseases, which are infectious and capable of causing economic, and biodiversity loss. To comply with the OIE guidelines to deal with the diseases, it is essential that a country has to develop disease information and reporting system built on a disease surveillance programme. In this direction, Aquatic Animal Disease Surveillance programme has been implemented in India since 2013 and diseases of concern are being listed. India also established its Aquatic Quarantine Facility (AQF) in the year 2009 at Chennai at the behest of Ministry of Agriculture for quarantining the *P. vannamei* broodstock imported to India. Establishment of this facility has boosted vannamei farming in India.

Aquaculture biosecurity

Biosecurity encompasses broad concepts of strategic and integrated approaches that include the policy and regulatory frameworks to analyse and manage risks in the sectors of food safety, life and health of animals and plants, including associated environmental risk. Application of biosecurity concepts to shrimp aquaculture will contribute significantly to reduce losses due to diseases and make this sector more sustainable and environmentally responsible. Biosecurity means "life protecting", but its use appears to be restricted to issues related to preventing the introduction, establishment or spread of unwanted biological organisms or agents. The principles of biosecurity should be considered to keep the pathogen not only out of the culture environment but also out of the country and the region. Implementation of biosecurity practices is an increasingly pressing issue for fisheries and aquaculture managers, considering the importance of this sector in terms of economic development of the people. Resource protection, food security, trade, production and development issues are driving this change (Beers, et al, 2005). Developing biosecurity programme includes identification of the species at-risk that is required to be protected by the programme, threats, pathways of hazards; assessment of the level of harm that would result; measures that could be used to mitigate the risk; documentation of the programme, its performance and auditing of the programme, preparation of contingency plans, and finally provision of adequate resources to implement the programme. The sanitary and phyto-sanitary measures (SPS

Agreement) provide an internationally enforceable set of rights and obligations on the use of biosecurity measures by governments.

A biosecurity programme is developed after scientific analysis of information with the aim of adopting procedures to manage risks to an acceptably low level. The use of sound epidemiological principles and a logical, structured approach will help in achieving the biosecurity. At farm level, implementing biosecurity plan requires modifying existing farms and management routines. In this instance, white spot disease (WSD) is taken as a case considering its devastating nature and biosecurity measures required for minimizing risks associated with this disease in aquaculture are outlined. Basic husbandry practices have to include elements of disease prevention and control. Transmission of viral diseases in aquaculture occurs through two pathways, viz., horizontally (transmitted by direct contact, or indirectly, by ingestion of infected organisms, and water), and vertically (virus is passed from an infected female parent to her progeny). A number of vectors such as rotifers, marine molluscs, polychaete worms and non-decapod crustaceans including *Artemia salina* and the copepods, as well as non-crustacean aquatic arthropods such as sea Slaters (Isopoda) and Euphydradae insect larvae can serve as host to this virus and transmit the disease in cultured stock. To date, more than 93 species of arthropods have been reported as hosts or carriers of WSSV either from culture facilities, the wild or experimental infection. Birds also can serve as potential sources of disease transmission. At present there is no treatment available to interfere with the unrestrained occurrence and spread of the disease. Biosecurity or pathogen exclusion from the rearing systems using highly sensitive diagnostic tools and adoption of better management practices involving rigorous sanitation practices have been helpful in prevention and control of the disease.

Preventing the entry of WSSV in hatcheries and grow-out phase of aquaculture can be achieved primarily using specific pathogen-free (SPF) or specific pathogen- resistant (SPR) and genetically improved (selective breeding method) stock. Specific pathogen-free history comes only from a long-term captive breeding and disease surveillance programme at a facility that has a fully functional and effective biosecurity plan. Pathogen carriers such as vectors, intermediate hosts, reservoir hosts, non-host biological carriers such as birds, insects, other predators, human beings and, fomites such as water, vehicles, buckets, shoes, nets, clothing also pose serious threat and management measures need to be incorporated to prevent entry of pathogens from these sources. This can be achieved using validated sensitive diagnostic tools.

Farm level of biosecurity measures have to be implemented by the farmers. Achieving biosecurity in hatcheries and farms requires preventing the entry of WSSV into hatcheries and farms, monitoring the health status of the shrimp population, adoption of better management practices including recommended protocols from using pathogen-free stock ascertained by robust diagnostics, pond preparation and management measures. Adopting quarantine measures for broodstock prior to their use, adopting better management practices (BMPs) and standard operating protocols (SOPs) by implementing good sanitary practices, treating water before use, optimizing stocking density of larvae and maintaining good water quality, treating hatchery effluent during seed production in hatchery will help in achieving biosecurity in hatcheries. Main preventive measures at pond / farm level include proper pond preparation to eliminate pathogens and their carriers, treatment of water in reservoirs to inactivate free viruses and kill virus carriers, water filtration using fine filters to keep carriers out, closed systems to avoid contamination from source water, reduced water exchange to minimize the entry from source water and even changing the water source. Transmission of virus can be prevented by providing crab fencing, fencing, foot baths, wheel baths, and disinfection protocols. Improved husbandry practices have been successfully employed for the control of diseases. Shrimp ponds with a history of disease outbreaks have a greater likelihood of future disease outbreaks, and hence, special attention is required during pond preparation in such farms.

International measures

The OIE (World Organisation for Animal Health) is an intergovernmental organization with 180 Member Countries is mandated under the World Trade Organization (WTO) Agreement on the Application of Sanitary and Phytosanitary Measures (SPS Agreement), to safeguard world trade by publishing health standards for international trade in animals and animal products. The OIE provides advisory and technical services through publication of *Aquatic Animal Health Code* and the *Manual of Diagnostic Tests for Aquatic Animals*. The aim of the *Aquatic Code* is to assure the sanitary safety of international trade in aquatic animals (fish, molluscs, and crustaceans) and their products. The *Aquatic Code* also provides a range of tools that assist Member Countries in preventing and controlling aquatic animal diseases. General tools range from recommendations on preventative approaches, such as import risk analysis (IRAs), through guidelines for contingency plans as part of the preparedness and response arrangements of a country, to recommendations on post-incident measures. The *Aquatic Code* provides guidelines on targeted surveillance for recognition of freedom from infection with a listed disease to help health certification prior to trade. The *Manual of Diagnostic Tests for Aquatic Animals* (the *Aquatic Manual*) provides a standardised approach to the diagnosis of the diseases listed in the *Aquatic Code*, to facilitate health certification for trade in aquatic animals and aquatic animal products.The *Aquatic Manual* is a key reference document describing the methods relevant to the OIE-listed diseases and other important diseases for use by aquatic animal health laboratories around the world.

Summary

Translocation of aquatic animals for diversification has emerged as a potential threat and source of new diseases. The strategy of the aquatic quarantine primarily involves the protection of our country's aquatic biodiversity from exotic organisms, pathogens and containment of the diseases. Controlling diseases in aquaculture is challenging as implementation of control measures is difficult, and resources for prevention are very limited. It has been realized that the best approach to managing aquatic animal health is to improve biosecurity at all levels. At present we can achieve biosecurity using validated highly sensitive diagnostic tools. Biosecurity provides a strategic framework and integrated approach to assess and manage the risks. Application of biosecurity concepts will contribute significantly to reduce losses due to diseases and make shrimp aquaculture sustainable and environmentally responsible.

Overview of diagnostics in aquacultureand PCR ring test

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The first scientific shrimp farming project started at Nellore, Andhra Pradesh (AP) through DBT funded project, executed by MPEDA, using hatchery produced tiger shrimp seed, formulated feed and paddle wheel aerators in 1990. This commercially successful demonstration project triggered the spread of shrimp farming on a commercial level in AP. Shrimp farming enterprise spread across AP and Tamilnadu (TN), through early nineties, until the emergence of the devastating white spot virus disease (WSD) in 1994, caused by white spot syndrome virus (WSSV). The lethal WSSV inflicted heavy crop losses and economic losses in the shrimp farming sector, year after year and the whole shrimp farming activity became an unsustainable enterprise.

Developments in application of molecular diagnostics in Indian aquaculture

Even today, more than two decades of emergence of WSD, there is no therapeutic option available, and the only option available is to detect and avoid the pathogen entry in the hatchery and farm, using smart diagnostic tools. An array of diagnostic tools has been developed for aquaculture use, mostly adapted from human and veterinary medicine. These include the first generation tools such as direct microscopy and histopathology, electron microscopy and conventional microbiology, followed by the second generation diagnostics: bioassay, tissue culture and immunoassays. The recent ones are the third generation DNA-based diagnostic tools, such as polymerase chain reaction (PCR) and DNA-hybridisation.What followed WSD emergence was a kind of diagnostic revolution, where PCR based WSSV detection labs were set up across AP and TN in private sector and the screening of broodstcoks and shrimp seeds were a routine. PCR methods were described since 1996 for the detection of WSSV and a number of diagnostic kits were marketed that cost farmers Rs.1200 to Rs.2000/- per test. Until 2008, majority of the farmers tested shrimp seed for WSSV in commercial PCR laboratories prior to stocking, and the practice helped to control the introduction WSSV in the rearing systems and managed successful crops using tiger shrimp *Peaneus monodon*.

Screening of viral pathogens through PCR laboratories by the farmers provided biosecurity in shrimp farms, preventing the spread of devastating infectious diseases such as WSD of shrimp through early detection and adoption of best management practices (BMPs). However, after the introduction of the exotic specific pathogen free (SPF) Pacific white shrimp, *Penaeus vannamei* in the year 2009, farmers gave away the practice of PCR screening of shrimp seed, possibly due to a wrong assumption of farmers that the seed originated from SPF stock, PCR testing was not required. As a fall out of this, many of the private laboratories became virtually non-functional as farmers did not come forward to test seed for WSD, and eventually closed down. Further, it is believed that some of the hatchery operators started practicing inbreeding of Pacific white shrimp for quick profit, ultimately losing the SPF status. What followed is a re-emergence of WSSV related crop losses along with new emerging disease situations such as running mortality syndrome (RMS), white faeces syndrome (WFS), stunted growth, white muscle syndrome (WMS) and the latest, shrimp hepatopancreatic microsporidosis caused by *Enterocytozoon hepatopenaei* (EHP) since 2013. This situation has therefore necessitated seed testing seed before stocking using smart DNA based diagnostic tools such as PCR.

Molecular diagnostics of shrimp diseases

Methods for diagnosis of diseases are evolving rapidly. There has been a rapid growth in the use of

molecular methods for aquatic animal diseases because, compared with many traditional methods, since they are more rapid, sensitive and cost effective. Commercial kits are becoming available for diagnosis of a number of aquatic animal diseases. The use of commercial kits is only acceptable if they have been validated as fit. The OIE provides guidance on the suitability of specific commercial kits through the Register of diagnostic kits certified by the OIE as validated and fit for purpose. Commercial kits included in the register have been evaluated in accordance with the Procedure for OIE Registration of Diagnostic Kits (http://www.oie.int/en/our-scientific-expertise/certification-of-diagnostic-tests/the-register-of-diagnostic-tests/). While the PCR based diagnosis of shrimp pathogens has been described elsewhere in this manual, an overview of molecular tools is provided in this section. The process of harmonisation of diagnostics through ring test is also provided in brief for the benefit of the laboratories participating in the ring test.

The effective control and treatment of diseases requires rapid, reliable and highly sensitive diagnostic tools. In many cases, post-mortem necropsy, histopathology and conventional microbiological isolation methods have been the primary methods for the diagnosis of fish and shellfish diseases. These methods are relatively less specific, time consuming and often inefficient to detect many pathogens when present in low numbers or when there are no clinical signs of disease. Efforts to overcome these problems led to the development of immunoassay and DNA-based diagnostic methods such as fluorescent antibody tests (FAT), enzyme-linked immunosorbent assays (ELISA), radioimmunoassay (RIA), *in situ* hybridization (ISH), dot blot hybridization DBH) and polymerase chain reaction (PCR) amplification techniques.

PCR based diagnostic methods used in aquaculture

Polymerase chain reaction (PCR) has been widely applied to the detection of shrimp viruses so that the risk of disease can be controlled. This includes screening of broodstock, larvae and post larvae in the hatchery and before stocking. PCR is also used for identifying carriers, checking water and sediment for viral contamination and monitoring health of shrimp in grow out ponds. PCR is a highly sensitive and robust technique for detection of shrimp pathogens. By definition, PCR is a nucleic acid amplification technique wherein a specific portion of nucleic acid from a target organism is amplified *in vitro*. Amplification is achieved using oligonucleotide primers that are specific for the portion of the DNA to be amplified. By designing oligonucleotide primers that are specific for an organism, it is possible to design PCR to amplify specifically DNA from any desired organism. The amplification requires the enzyme DNA polymerase, and the building blocks of DNA, the deoxyribonucleotides. The reaction is performed in several cycles, each cycle consisting of three steps DNA denaturation, primer annealing, primer extension. Normally about 30 cycles of reaction are performed. Since each cycle involves denaturation of DNA at 95°C, the DNA polymerase used in the reaction should be thermostable. The discovery of thermostable DNA polymerase from the thermophilic bacterium *Thermus aquaticus* led to rapid application of PCR in diagnostics. The sensitivity of PCR could be further improved by performing nested PCR. The first primer set amplifies the target sequence, which then serves as the template for a second amplification. The second primer set lies internal to the first target sequence. Secondary amplification will not occur if the primary amplification did not happen.

For the detection of RNA viruses, the RNA target as of a RNA virus is first converted into a complementary DNA (cDNA) by the reverse transcriptase enzyme. This cDNA is used as template and amplified by standard PCR methods. Reverse-transcription PCR is used not only to detect pathogens, but also to detect the specific expression of certain genes during the course of growth or infection since they are amplified at a much higher number of messenger or ribosomal RNA than the number of DNA copies. In contrast to the detection of DNA from nonviable organisms using standard PCR, the detection of cDNA from messenger RNA encoded by a pathogen using

reverse-transcription PCR could be evidence of active infection.

Real-time PCR, described in detail elsewhere in this manual, is used to amplify and simultaneously quantify a targeted DNA molecule. This enables detection and quantification of the viral pathogen in the tissues of infected shrimp. It offers continuous monitoring of PCR product formation throughout the reaction and eliminates post-PCR analysis process. Thus, it shortens detection time compared to standard PCR. The viral load in infected shrimp can be accurately determined. Four types of indicators such as TaqMan probes, SYBR Green dyes, molecular beacons, fluorescence resonance energy transfer (FRET) hybridization probes are used frequently in real-time PCR methods for pathogen detection.

What is ring test / harmonization of PCR diagnosis?

Lack of uniform laboratory results and consensus among PCR laboratories has necessitated greater attention to standardization and harmonization, during the post WSSV period since 1996. One of the purposes of inter laboratory performance testing, also known as a ring test, is to assure the farmers or regulatory officials that the results provided are accurate and specific, so that farmers could make the seed selection based on the results of PCR test, conducted at the private PCR labs. Another purpose is to determine if the test methods in use are reliable and reproducible. Participation in the ring testing is completely voluntary, and PCR labs have their choice in the use of methods and test kits, and only outcome of the test results in terms of sensitivity and reproducibility are evaluated. Each laboratory is provided with a panel of four coded tissue samples (for one pathogen) fixed in 95% ethanol for testing. A standard report format is provided, and the laboratories are given a maximum of three working days to analyse the samples. For the reports to be considered complete, the primer sets or commercial kits, extraction methods, PCR conditions and gel photographs must be included in the participating labs' reports. Laboratories which employ real-time PCR are expected to include their chromatograms in the results section of the report. The results from each laboratory are evaluated and summarised and compiled into a report and distributed to the participants. Results are kept confidential, and only provided to the participants through the specific codes which are known to only individual laboratories and the nodal institute, ICAR-CIBA. Since the purpose of the ring test is not only to determine proficiency, but to help improve performance, feedback is also provided to those laboratories experiencing problems with the analysis, so that they get an opportunity to provide a quality service to the farmer.

WSSV PCR Ring tests in 2005 and 2008

The first ever PCR ring test was conducted in India at ICAR-CIBA, Chennai in the year 2005 under a regional project titled *"Application of PCR for improved shrimp health management in the Asian region"* implemented in India, Indonesia, Thailand and Australia, with key partners including ACIAR, CSIRO and AusVet Services in Australia; MPEDA, CIBA and College of Fisheries, Mangalore in India; Mahidol University, BIOTEC and FAO-NACA in Thailand; Ministry of Marine Affairs and Fisheries in Indonesia. 46 applications were received from private and government PCR service providing labs, hatcheries and research institutions, out of which, 21 laboratories successfully completed the PCR ring test, which were available across the shrimp farming regions of AP and TN. The second voluntary PCR ring testing exercise was conducted in the year 2009 by ICAR-CIBA, in which 34 laboratories participated and 31 participants provided their results. 21 laboratories successfully completed the 2nd PCR ring testing exercise in the year 2009.

Purpose of Ring test 2016

The work carried out at CIBA under the National Surveillance Programme for aquatic Animal Diseases (NSPAAD) in India funded by the National Fisheries Development Board (NFDB) has indicated emergence of a new pathogen, *Enterocytozoon hepqtopenaei* (EHP), and has been responsible for causing stunted growth in farmed shrimp and thus reducing productions. Further, the

study has also indicated that WSD caused by WSSV continues to be the major killer of farmed shrimp.Since 2009, the newly emerging disease such as early mortality syndrome (EMS), specifically known as Acute Hepatopancreatic Necrosis Disease (AHPND), has caused large losses among shrimp farmers in China, Vietnam, Malaysia and Thailand. Considering the limited therapeutic options available for the control of diseases, only timely disease detection using novel diagnostic tools for disease surveillance followed by active response to adopt and practise proper health management approaches would ameliorate the magnitude of the problem. The PCR diagnostic testing does require significant attention and validation to ensure greatest possible sensitivity and specificity, so that credible PCR testing services could be made available for the farmers.

CIBA initiated the process of ring test involving the Coastal Aquaculture Authority (CAA, regulatory body) and Rajiv Gandhi Centre for Aquaculture (RGCA) under the Marine Products Export s Development Authority (Ministry of Commerce's promotional agency for shrimp farming). Expression of interest (EOI) for participation in the ring test was invited by CAA and widely publicised through advertisements in newspapers and on websites of CAA, CIBA and RGCA. The exercise is proposed to be carried out in the following three stages.

Stage I: PCR infrastructure available with laboratories: laboratory layout and availability of equipment; inspection by team from CIBA and RGCA, method of inspection and reporting (for inspection team).

Stage II: Training at CIBA for lab technicians: The PCR training for three days would cover theoretical and practical classes on nucleic acids, principles and practice of polymerase chain reaction (PCR); a detailed discussion on the various diagnostic tests used for white spot syndrome virus (WSSV), infectious hypodermal and hematopoietic necrosis virus (IHHNV), microsporidian parasite *Enterocytozoan hepatopenaei* (EHP) and acute hepatopancreatic necrosis disease (AHPND).

Stage-III: Ring test-sample distribution; submission of results by the participating laboratories and evaluation of the ring test.

The successful laboratories would be issued with certificates. The laboratories failing to pass would be provided additional training at CIBA including trouble-shooting the issues in PCR diagnostics and reporting.

The aquaculture sector in India growing and theuse ofpathogen free quality seed is an important factor in the success and sustainability of shrimp farming in India, and this has been putting across the stake holders and farmers by theR&D institutions such CIBA and govt. arms such as DADF, NFDB and CAA. The number of quality diagnostic service laboratories has to increase in the private sector to cater to the needs of the growing shrimp farming sector. Ring testing and harmonisation of diagnostic services has to be a continuous exercise. Further, farmers often look forward to a Govt. Referral Laboratory for validating the results provided by the private laboratories. Presently CIBA is carrying out the diagnostics on samples coming through the DADH&F, MoA (OIE listed pathogens in internationally traded fish and fishery products on all quarantine products related to brackishwater aquaculture. The success of shrimp farming depends on the right choice and use of the diagnosticsby the aquaculturists to make rapid, prudent and cost-effective management decisions. Diagnostic tools are not a solution, but the means for an effective aquaculture health management.

OIE listed shrimp diseases of shrimp: An Overview

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Introduction

Aquaculture is one of the world's fastest growing industries in food production. Disease outbreaks cause significant losses in aquaculture production and trade and are having an impact on the economic development of some countries. An indication of the magnitude of economic losses is illustrated by farm surveys conducted in sixteen Asian countries, which show that annual losses due to disease in the region total more than USD 3 billion. Probably the most striking example of disease spread through international trade and consequential major economic loss in aquaculture is white spot disease (WSD) in farmed shrimp. The disease first emerged in 1991 in a shrimp farm in an OIE Member Country and apparently has since spread to most other shrimp-farming countries. The Global Outlook for Aquaculture Leadership (GOAL) 2014 survey reported substantial production declines during 2012-2013 in China, Thailand, Vietnam and Mexico as a consequence of the Early Mortality Syndrome (EMS) disease that originated in China in 2009.

The increasing trend in intensification and commercialization of aquaculture has exacerbated the epidemics and have become a major constraint for the economic viability of this industry. Severe disease related mortality and thereby economic loss due to different viral agents such as monodon baculovirus (MBV) in Taiwan, infectious hypodermal and hematopoietic necrosis virus (IHHNV) in the Americas, yellow head virus (YHV) in Thailand and Taura syndrome virus (TSV) in the Americas during the different periods have been reported. In addition to these, the major disease outbreak due to White Spot Syndrome Virus (WSSV) has also been reported from all parts of the world. During the recent past, the havoc created by Early Mortality Syndrome (EMS) in different South East Asian countries has also been found to have severe economic impact on shrimp industry of these regions. Further, a number of other diseases by unidentified etiologies are constantly being associated with shrimp culture practice which has been responsible either for direct mortality or growth reduction and thereby bringing loss to farmers. This section is aimed at highlighting the common shrimp diseases and their management with special reference to OIE listed diseases.

White spot syndrome

White spot syndrome (WSD) is the most devastating diseases of penaeid shrimp and is caused by white spot syndrome virus (WSSV), a rapidly replicating and highly virulent shrimp virus that has wide spread presence throughout the world. It is one of the largest viruses designated as genus *Whispovirus* in the family *Nimaviridae*. WSSV is a rod-shaped, double-stranded, DNA virus, and the size of the enveloped viral particles have been reported to be 240 -380 nm long and 70 -159 nm in diameter. Originating from Taiwan in 1992, it spread to Japan during 1993 and subsequently very quickly to all other Asian countries. By 1995, it had already spread to North America and further by 1999 to South America. The quick spread of this virus to different regions and simultaneous investigation by different scientists speculated the same agent to be different ones and thereby called them in different names such as systemic ectodermal and mesodermal baculovirus (SEMBV), rod shaped nuclear virus of *Penaeus japonicus* (RV-Pj), hypodermal and haematopoietic necrosis baculovirus (HHNBV), third *Penaeus monodon* non-occluded virus (PmNOB III), penaeid rod shaped DNA virus or white spot baculovirus. On subsequent investigation and data analysis when it was known that all these names are for the same agent, it was unanimously called as white spot syndrome virus. Other than the penaeid shrimps which serve as host for this virus, a large number of other crustaceans

serve as carrier and therefore it has been impossible to eradicate this virus from the culture system. The typical clinical symptoms of WSSV infection include loose cuticle, often with reddish discolouration, and the presence of white spots of 0.5 to 2.0 mmin diameter on the inside surface of the carapace, appendages and cuticle over the abdominal segments. Outbreak of this disease wipes out the entire shrimp populations in the ponds within 3-8 days.

Histopathology can be observed inthegillepithelium,antennalgland,haematopoeitic tissue,nervous tissue,connective tissueand intestinalepithelium.Pathogenesis involves widespread tissue necrosis and disintegration. Infected cells exhibit prominent intranuclear occlusions that initially stain eosinophilic, but become basophilicwith time; hypertrophied nuclei with chromatin margination and cytoplasmic clearing.Rapid and specific diagnosis of the WSD can be accomplished using nested or quantitative polymerase chain reaction.

WSSV is transmitted vertically from infected broodstock to larvae and horizontally by ingestion of infected organisms. While WSD transmission vertically is being prevented by screening out infected broodstock, its horizontal transmission in grow out farms is a serious challenge. At present there is no treatment available to prevent the unrestrained occurrence and spread of the disease. Better management practices (BMPs) have helped alleviate this problem to a great extent, by minimising risks of its transmission through carrier organisms such as mud crabs,*Artemia*, rotifer eggs, molluscs, polychaete worms, insect larvae and seabirds etc. However, concerns of WSD transmission through contaminated water and pond sediment remain unaddressed. Pond preparation practices have proved to be useful in eliminating the virus from the pond and reducing the risk of disease outbreaks.

Yellow Head Disease (YHD)

Yellow head disease was the first major viral disease that caused extensive losses to shrimp farms in Thailand during 1990-91. YHD outbreaks have been reported in the black tiger prawn and the white Pacific shrimp. YHD has been reported in China, Taipei, India, Indonesia, Malaysia, the Philippines, Sri Lanka, Thailand and Vietnam. Outbreaks of YHD with heavy mortalities have been reported in farmed black tiger shrimp and pacific white shrimp. It is reported to be highly prevalent (>50%) sampled farmed and wild populations in Australia, Asia, East Africa and Mexico.YHD is caused by yellow head virus (YHV), and its close relatives such as gill-associated virus (GAV). YHV is rod-shaped enveloped viruses of 40-60 nm by 150-200 nm size, containing single stranded RNA. YHV affects tissues of ectodermal and mesodermal origin including lymphoid organ, haemocytes, haematopoietic tissue, gill lamellae and spongy connective tissue of the sub-cutis, gut, antennal gland, gonads, nerve tracts and ganglia. YHV principally affects pond reared juvenile stages of 5-15 g. Affected shrimp typically feed voraciously for two to three days and then stop feeding abruptly and are seen swimming near the periphery of the pond. YHV infections can cause swollen and light yellow colored hepatopancreas in infected shrimp, and a general pale appearance, before dying within a few hours. YHD can cause up to 100% mortality in infected *P. monodon* ponds within 3-5 days of the first appearance of clinical signs. GAV has been reported to be associated with mortalities of up to 80% in *P. monodon* ponds in Australia.Yellow head virus can be detected by RT-PCR or with a probe designed for dot-blot and *in situ* hybridization tests. It can also be diagnosed histologically in moribund shrimp by the presence of intensely basophilic inclusions, most easily in H&E stained sections of stomach or gill tissue, or simply by rapid fixation and staining of gill tissue and microscopic examination. The primary mechanism of spread of YHV in pond culture appears to be through water and mechanical means. Infected broodstock can pass on the virus to larvae in the maturation/hatchery facilities if thorough disinfection protocols are not strictly adhered to. Methods of YHV eradication in ponds are much the same as for other viruses and involve BMPs that include pond preparation by disinfection and

elimination of carriers and production of virus free broodstock and PL for pond stocking.

Taura Syndrome (TS)

Taura Syndrome was first identified from farms around the Taura River in Ecuador in 1992 and the disease spread rapidly to the whole of Latin and North America within three years. Subsequently, TS was also reported from Asia including Mainland China and Taiwan (from 1999), and in late 2003 in Thailand, probably through the regional and international transfer of live PL and broodstock of *L.vannamei*. Initial work suggested that TS was caused by a toxic pesticide. However, it is now knownthat a single or perhaps several very closely related strains (mutants) of the Taura syndrome virus(TSV) are responsible for the TS. TSV is a single stranded RNA virus of 32 nm size, non-enveloped icosahedrons and more prone to mutations causing more concern.

TSV infections occur in juvenile shrimp (0.1-1.5 g body weight) within two to four weeks of stocking ponds and occur largely within the period of a single moult cycle. In the acute phase of the disease, during pre-moult stage, the shrimp are weak, soft-shelled, have empty gut and diffuse expanded chromatophores that appear red, particularly in the tail (hence the common name - red tail disease). Such animals will usually die during moulting (5-95%). Adult shrimp are known to be more resistant than juveniles. Shrimp that survive infection show signs of recovery and enter the chronic phase of the disease. Such shrimp show multiple, randomly distributed, irregular, pitted, melanised lesions of the cuticle. These gross lesions will persist, but may be lost during moulting, and the shrimp thereafter appear normal. TS can be diagnosed using standard histological and molecular methods of detection. Specific DNA probes applied to *in situ* hybridization assays with paraffin sections provide the confirmatory diagnosis. Reverse transcriptase polymerase chain reaction (RT-PCR) assay is commonly used for larger sample sizes and non-lethal sampling for broodstock. Histological demonstration of enlarged lymphoid organs (LO) with multiple LO spheroids and multifocal areas of necrosis in the cuticular epithelium of the general body surface, appendages, gills, hindgut, and foregut (the oesophagus, anterior and posterior chambers of the stomach).The mechanism of transmission of TSV can be through contaminated PL and broodstock. Recently it has been shown that mechanical transfer through insect. The disease can be prevented by avoidance of reintroduction of the virus from wild shrimp and carriers and stocking with TSV-free PL produced from TSV-free broodstock.

Infectious Hypodermal and Haematopoietic Necrosis (IHHN)

IHHN was first discovered in *L. vannamei* and *P. stylirostris* in the Americas in the year 1981. However, it was thought to have been introduced along with live *P. monodon* from Asia. IHHNV has probably existed for some time in Asia without detection due to its insignificant effects on *P. monodon*, the major cultured species in Asia. Recent studies have revealed geographic variations in IHHNV isolates, and suggested that the Philippines was the source of the original infection in Hawaii, and subsequently in most shrimp farming areas of Latin America. Large-scale epizootics were responsible for multi-million dollar losses in *L. vannamei* culture in the Americas during the 1990s. IHHNV is caused by a small (20-22 nm) single-stranded DNA-containing parvovirus. Gross signs of disease are not specific to IHHN, but may include reduced feeding, elevated morbidity and mortality rates, fouling by epicommensals and bluish body coloration. Larvae, PL and broodstock rarely show symptoms. In *L. vannamei*, IHHNV can cause runt deformity syndrome (RDS), which typically results in cuticular deformities (particularly bent rostrums), slow growth, poor FCR and a greater size variation at harvest, contributing substantially to reduction in profits. IHHNV typically causes no problems for *P. monodon* since they have developed a tolerance to it over a long period of time, but they may suffer with RDS. *P. merguiensis* and *P. indicus* appear refractory to the IHHNV. However, these species may be life-long carriers of the virus and so could easily pass it onto *L. vannamei*, which typically

suffer from RDS when exposed to IHHNV.IHHNV can be diagnosed using methods such as DNA probes in dot blot and *in situ* hybridization and PCR techniques (including real-time PCR) as well as histological analysis of H&E-stained sections looking for intracellular, Cowdry type A inclusion bodies in ectodermal and mesodermal tissues such as cuticular epithelium, gills, foregut, hind gut, lymphoid organ and connective tissues. Transmission of IHHN is known to occur rapidly by cannibalism shrimp. It can also be transmitted through waterborne route and cohabitation. Vertical transmission from broodstock to larvae is common. Strict hatchery biosecurity including checking of broodstock by PCR, or the use of SPF broodstock, washing and disinfecting of eggs and nauplii is essential in combating this disease.

Infectious Myonecrosis (IMN)

Infectious myonecrosis is an emerging *L. vannamei* disease, first detected in Brazil during 2004, and then in Indonesia in 2006. To date, IMN has been detected in East Java, Bali, and West Nusa Tenggara provinces. The principal host species in which IMNV is known to cause significant disease outbreaks and mortalities is *L. vannamei*. IMN is caused by a virus, a putative totivirus. IMNV particles are icosahedral in shape and 40 nm in diameter. Juveniles and sub-adults of *L. vannamei*, farmed in marine or low salinity brackish water, appear to be the most severely affected by IMN disease. The principal target tissues for IMNV include the striated muscles (skeletal and less often cardiac), connective tissues, haemocytes, and the lymphoid organ parenchymal cells. IMN disease causes significant mortality in grow-out ponds and is characterized by acute onset of gross signs including focal to extensive whitish necrotic areas in the striated muscle, especially of the distal abdominal segments and the tail fan, which may become necrotic and reddened similar to the colour of cooked shrimp. Severely affected shrimp become moribund and mortalities can be instantaneously high and continue for several days. Mortalities from IMN range from 40 to 70% in cultivated *L. vannamei*, and food conversion ratios (FCR) of infected populations increase from normal values of ~ 1.5 to 4.0 or higher. IMN can be confirmed by histopathology, using routine haematoxylin-eosin (H&E) stained paraffin sections and demonstrating characteristic coagulative necrosis of striated skeletal muscle fibres, often with marked oedema among affected muscle fibres. IMN may be also rapidly diagnosed using a nested reverse-transcriptase polymerase chain reaction (RT-PCR) method which provides a rapid, sensitive and specific test to detect IMNV in penaeid shrimp. Published methods are available for the molecular detection of IMNV by *in-situ* hybridisation (ISH), nested RT-PCR and real-time RT-PCR. IMNV has been demonstrated to be transmitted through cannibalism. Transmission via water and vertical transmission from broodstock (transovarian or by contamination of the spawn eggs) to progeny is also likely to occur. IMNV may also be transmitted among farms by faeces of seabirds or shrimp carcasses. Outbreaks of IMN with sudden high mortalities may follow stressful events such as capture by cast-net, feeding, sudden changes in salinity or temperature, etc., in early juvenile, juvenile, or adult *L. vannamei* in regions where IMNV is enzootic. Stocking only pre-screened broodstock and/or their spawned eggs/ nauplii and discarding those that test positive for the IMN virus by reverse-transcription polymerase chain reaction (RT-PCR). The disease can be prevented by stocking with virus free PL produced from IMNV-free broodstock.

Necrotizing Hepatopancreatitis (NHP)

This disease is also known as Texas necrotizing hepatopancreatitis (TNHP), Texas pond mortality syndrome (TPMS), and Peru necrotizing hepatopancreatitis (PNHP). NHP has been reported as an important disease since its first diagnosis in 1985. It has been reported to cause mass mortalities to the tune of 20-90 percent of *L. vannamei* in highly saline commercial grow-out ponds nearly every year since then. By 1993, NHP spread to Ecuador, Guatemala, Honduras, Mexico and Peru and by 1995, coincided with warm waters with high salinity associated with El Nino, and caused severe mortalities (60-80 percent mortality) of *L. vannamei* and *L. stylirostris* throughout

Ecuador. NHP has not yet been reported in Asia, but could cause significant damage were it to be transferred here with untested shrimp introduction. Necrotizing hepatopancreatitis is caused by obligate intracellular Rickettsia-like bacterium, a member of the order a-Proteobacteria (Gram-negative, pleomorphic, rod-shaped or helical-shaped bacterium). Affected shrimp are lethargic, anorexic with empty gut and show epibiotic fouling. Exoskeleton becomes soft and show abdominal muscle atrophy. Affected ponds have increased FCR and growth of affected shrimp is retarded. The hepatopancreas becomes watery with white or black streaks. Mortality rates reach up to 90% within 30 days of the appearance of clinical signs. NHP can be diagnosed by demonstration of lipid droplets and melanisation of hepatopancreas by microscopic examination of wet mount of preparations. It may be confirmed by histopathologicalexamination showing atrophy and the presence of granuloma in the hepatopancreas, and haemocyte aggregations around the hepatopancreatic tubules. Intra-cytoplasmic Rickettsia-like bacteria may be prominently seen in the cytoplasm. Molecular diagnostic tools such as *in situ* hybridization, dot blot hybridisation, and PCR for specific a-Proteobacterial DNA are also available. NHP could be transmitted horizontally with infected PLs. maintaining optimal environmental parameters using BMPs will be useful in preventing NHP.

Since 2009, an emerging threat, popularly known as early mortality syndrome (EMS) and scientifically termed as acute hepatopancreatic necrosis disease (AHPND) severely affected shrimp farming in many countries in the Southeast Asian region. Though the disease is reported to affect mainly Pacific white shrimps (*Penaeus vannamei*), tiger shrimp (*Penaeus monodon*) and Chinese shrimp (*Penaeus chinenesis*) have also been reported to be susceptible. The disease is mainly characterized by mass mortalities (reaching up to 100% in many cases) during the first 20-35 days of culture (post-stocking in grow-out ponds). This new disease was first reported from China in 2009, followed by Vietnam in 2010, Malaysia in 2011, Thailand in 2012, and Mexico in 2013.Very recently during 2015, this disease has also been reported to be present in the Philippines. The disease has caused severe economic losses throughout the region. AHPND has been recently listed by the OIE and is reportable since 2016.

The early mortality syndrome in shrimp has been named based on unusually high mortality within the first 30 days of shrimp grow-out culture, due to a variety of pond management and pathogen related factors. Some of the farm level clinical signs includeonset of clinical signs such as soft shells, significantly emaciated pale to whitish hepatopancreas (HP) and partially full to empty guts and mortality starting as early as 10 days post stocking. HP is the main target in AHPND and therefore the pond level observations have to be further confirmed as AHPND by laboratory investigations involving characteristic pathology of HP such as necrosis of B, F, R cells and sloughing of cells into the lumen. Though histo-pathological investigations are the gold standard for the confirmation and presence of AHPND, recently detection of the causative agent of AHPND by molecular methods such as PCR has been developed. The causative agent of the disease is a special strain of *Vibrio parahaemolyticus* (VPAHPND). A plasmid (pAP1) of about 69 kb present in this specific strain contains two genes that produce toxins (Pir A/B) and cause massive damage to the hepatopancreatic cells. AHPND/EMS is caused by an infectious agent, particularly by a specific strain of a bacterium that is commonly found in the shrimp culture environment. Adoption of biosecurity measures to prevent this pathogen entering the culture system.

Nucleic Acids

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Genetic material

Our concept of the gene has undergone a remarkable evolution as biologists have learned more and more about the nature of inheritance. The earliest studies revealed genes to be discrete factors that were retained throughout the life of an organism and then passed on to each of its progeny. Over the following century, these hereditary factors were shown to reside on chromosomes and to consist of DNA, a macromolecule with extraordinary properties. Figure 1 provides an overview of some of the early milestones along this remarkable journey of discovery, capped by the description of the double helical structure of DNA in 1953. In the decades that followed this turning point, a major branch of molecular biology began to focus on the genome, which is the collective body of genetic information that is present in a species. A genome contains all of the genes required to "build" a particular organism. Life depends on the ability of cells to store, retrieve, and translate the genetic instructions required to take and maintain a living organism. This hereditary information is passed on from a cell to its daughter cells at cell division, and from one generation of an organism to the next through the organism's reproductive cells. The instructions are stored within every living cell as its genes, the information-containing elements that determine the characteristics of a species as a whole and of the individuals within it.

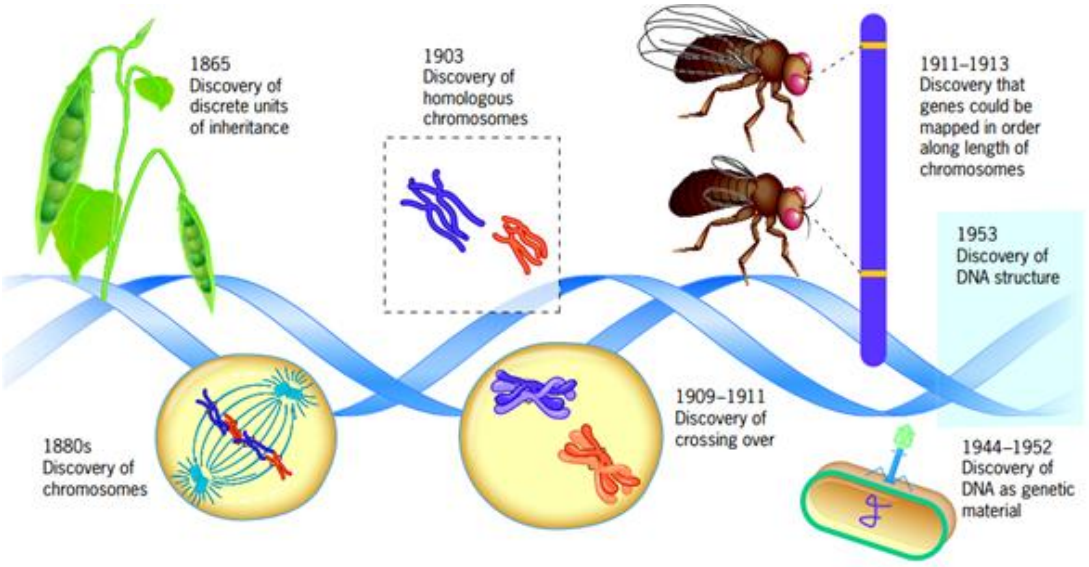

Fig. 1. An overview depicting several of the most important early discoveries on the nature of the gene. *Cell and Molecular Biology – Gerald Karp*.

Nucleic acid is the Carrier of Genetic Information

When biochemical analysis became possible, chromosomes were found to consist of deoxyribonucleic acid (DNA) and protein, with both being present in roughly the same amounts. For many decades, the DNA was thought to be merely a structural element. However, the other crucial advance made in the 1940s was the identification of DNA as the likely carrier of genetic information. This breakthrough in our understanding of cells came from studies of inheritance in bacteria (Figure 2). These experiments, carried out in the 1920s (A) and 1940s (b), showed that adding purified DNA to a bacterium changed the bacterium's properties

and that this change was faithfully passed on to subsequent generations. Two closely related strains of the bacterium Streptococcus pneumoniae differ from each other in both their appearance under the microscope and their pathogenicity. One strain appears smooth (S) and causes death when injected into mice, and the other appears rough (R) and is nonlethal. (A) An initial experiment shows that some substance present in the S strain can change (or transform) the R strain into the S strain and that this change is inherited by subsequent generations of bacteria. (b) This experiment, in which the R strain has been incubated with various classes of biological molecules purified from the S strain, identifies the active substance as DNA.

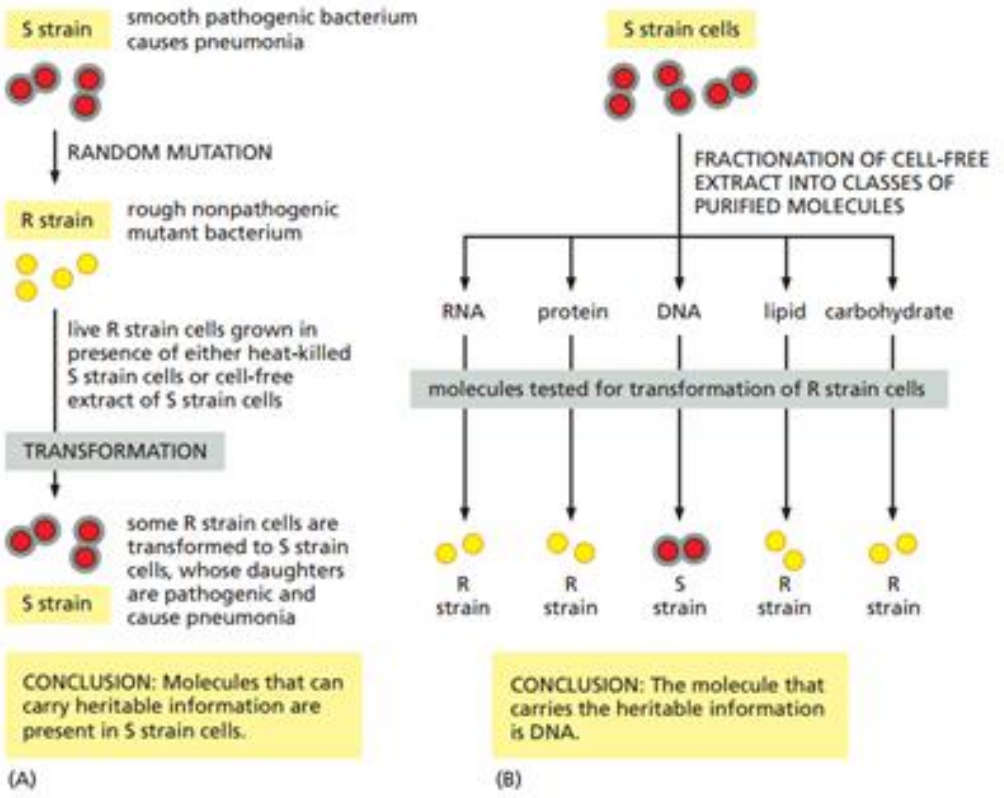

Fig.2. The first experimental demonstration that DNA is the genetic material.*Molecular biology of the Cell – Bruce Alberts.*

The Structure of DNA

To understand the workings of a complex macromolecule, whether it is a protein, polysaccharide, lipid, or nucleic acid and it is essential to know how that molecule is constructed. The mystery of DNA structure was investigated by a number of laboratories in both the United States and England in the early 1950s and was solved by James Watson and Francis Crick at Cambridge University in 1953.

- Deoxyribonucleic acid (DNA), the genetic material, carries information to specify the amino acid sequences of proteins. It is transcribed into several types of ribonucleic acid (RNA), including messenger RNA (mRNA), transfer RNA (tRNA), and ribosomal RNA (rRNA), all of which function in protein synthesis.

- All DNAs and most RNAs are long, unbranched polymers of nucleotides. A nucleotide consists of a phosphorylated pentose linked to an organic base, either a purine or a pyrimidine.

- The purines adenine (A) and guanine (G) and the pyrimidine cytosine (C) are present in both DNA and RNA. The pyrimidine thymine (T) present in DNA is replaced by the pyrimidine uracil (U) in RNA.

- Adjacent nucleotides in a polynucleotide are linked by phosphodiester bonds. The entire strand has a chemical directionality with 5′ and 3′ ends.

- Natural DNA (B DNA) contains two complementary antiparallel polynucleotide strands wound together into a regular right-handed double helix with the bases on the inside and the two sugar-phosphate backbones on the outside.Base pairing between the strands and hydrophobic interactions between adjacent base pairs stacked perpendicular to the helix axis stabilize this native structure.

- The bases in nucleic acids can interact via hydrogen bonds. The standard Watson-Crick base pairs are G·C, A·T (in DNA), and G·C, A·U (in RNA). Base pairing stabilizes the native three-dimensional structures of DNA and RNA.

- Binding of protein to DNA can deform its helical structure, causing local bending or unwinding of the DNA molecule.

- Heat causes the DNA strands to separate (denature). The melting temperature (Tm) of DNA increases with the percentage of G·C base pairs. Under suitable conditions, separated complementary nucleic acid strands will renature.

The Importance of the Watson-Crick Proposal

1. Storage of genetic information. As genetic material, DNA must contain a stored record of instructions that determine all the inheritable characteristics that an organism exhibits. In molecular terms, DNA must contain the information for the specific order of amino acids in all the proteins that are synthesized by an organism.

2. Replication and inheritance. DNA must contain the information for synthesis of new DNA strands (replication). DNA replication allows genetic instructions to be transmitted from one cell to its daughter cells and from one individual to its offspring.

3. Expression of the genetic message. DNA is more than a storage center; it is also a director of cellular activity. Consequently, the information encoded in DNA has to be expressed in some form that can take part in events that are taking place within the cell. More specifically, the information in DNA must be used to direct the order by which specific amino acids are incorporated into a polypeptide chain.

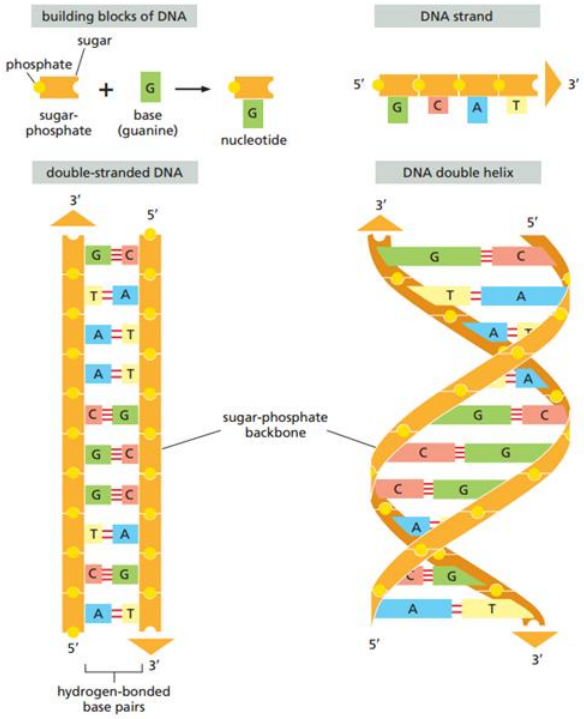

Fig.3.DNA and its building blocks

DNA is made of four types of nucleotides, which are linked covalently into a polynucleotide chain (a DNA strand) with a sugar-phosphate backbone from which the bases (A, C, G, and T) extend. A DNA molecule is composed of two antiparallel DNA strands held together by hydrogen bonds between the paired bases. The arrowheads at the ends of the DNA strands indicate the polarities of the two strands. In the diagram at the bottom left of the figure, the DNA molecule is shown straightened out; in reality, it is twisted into a double helix, as shown on the right.

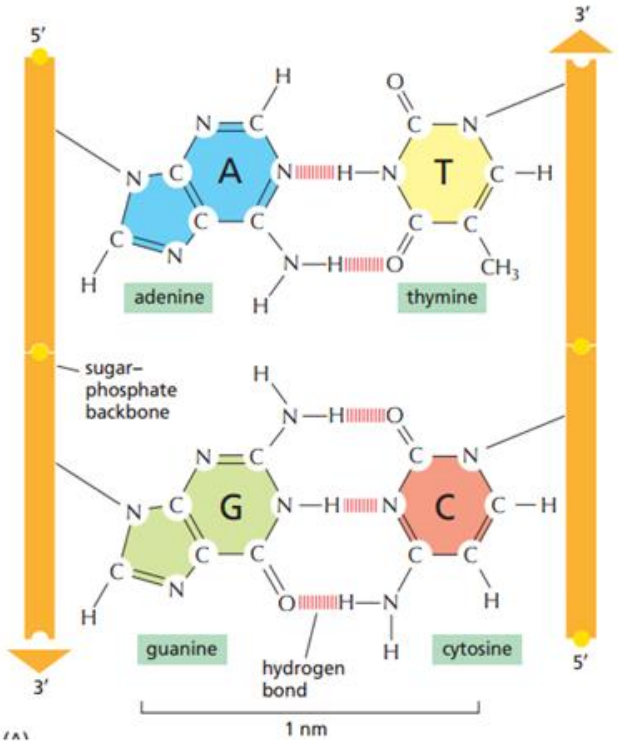

Fig. 4.Complementary base pairs in the DNA double helix.

The shapes and chemical structures of the bases allow hydrogen bonds to form efficiently only between A and T and between G and C, because atoms that are able to form hydrogen bonds can then be brought close together without distorting the double helix. As indicated, two hydrogen bonds form between A and T, while three form between G and C. The bases can pair in this way only if the two polynucleotide chains that contain them are antiparallel to each other.

DNA Denaturation, As first suggested by Watson and Crick, the two strands of a DNA molecule are held together by weak, noncovalent bonds. When DNA is dissolved in saline solution and the solution is slowly warmed, a temperature is reached when strand separation begins. Within a few degrees, the process is generally complete and the solution contains single-stranded molecules that are completely separated from their original partners. The progress of thermal denaturation (or DNA melting) is usually monitored by following the increase in absorbance of ultraviolet light by the dissolved DNA. The temperature at which the shift in absorbance is half completed is termed the melting temperature (Tm). The higher the GC content (%G + %C) of the DNA, the higher the Tm.

DNA Renaturation The separation of the two strands of the DNA duplex by heat is not an unexpected finding, but the re-association of single strands into stable double-stranded molecules with correct base pairs seems almost inconceivable. However, in 1960, Julius Marmur and Paul Doty at Harvard University found that if they slowly cooled a solution of bacterial DNA that had been thermally denatured, the DNA regained the properties of the double helix; it absorbed less ultraviolet light and once again behaved like genetic material in being able to transform bacterial cells. It became apparent from these studies that complementary single-stranded DNA molecules were capable of re associating, an event termed renaturation, or reannealing. This finding has proved to be one of the most valuable observations ever made in molecular biology. On one hand, reannealing has served as the basis for an investigation into the complexity of the genome, a subject discussed in the following section. On the other hand, reannealing has led to the development of a methodology called nucleic acid hybridization, in which complementary strands of nucleic acids from different sources can be mixed to form double-stranded (hybrid) molecules.

Structure of RNA

The primary structure of RNA is generally similar to that of DNA, with two exceptions: the sugar component of RNA, ribose, has a hydroxyl group at the 2′ position and thymine in DNA is replaced by uracil in RNA. The presence of thymine rather than uracil in DNA is important to the long-term stability of DNA because of its function in DNA repair. As noted earlier, the hydroxyl group on the 2′ carbon of ribose makes RNA more chemically labile than DNA. As a result of this lability, RNA is cleaved into mononucleotides by an alkaline, whereas DNA is not. The 2′-hydroxyl group of RNA also provides a chemically reactive group that takes part in RNA mediated catalysis. Like DNA, RNA is a long polynucleotide that can be double-stranded or single-stranded, linear or circular. It can also

participate in a hybrid helix composed of one RNA strand and one DNA strand. As mentioned above, RNA-RNA and RNA-DNA double helices have a compact conformation like the A form of DNA.

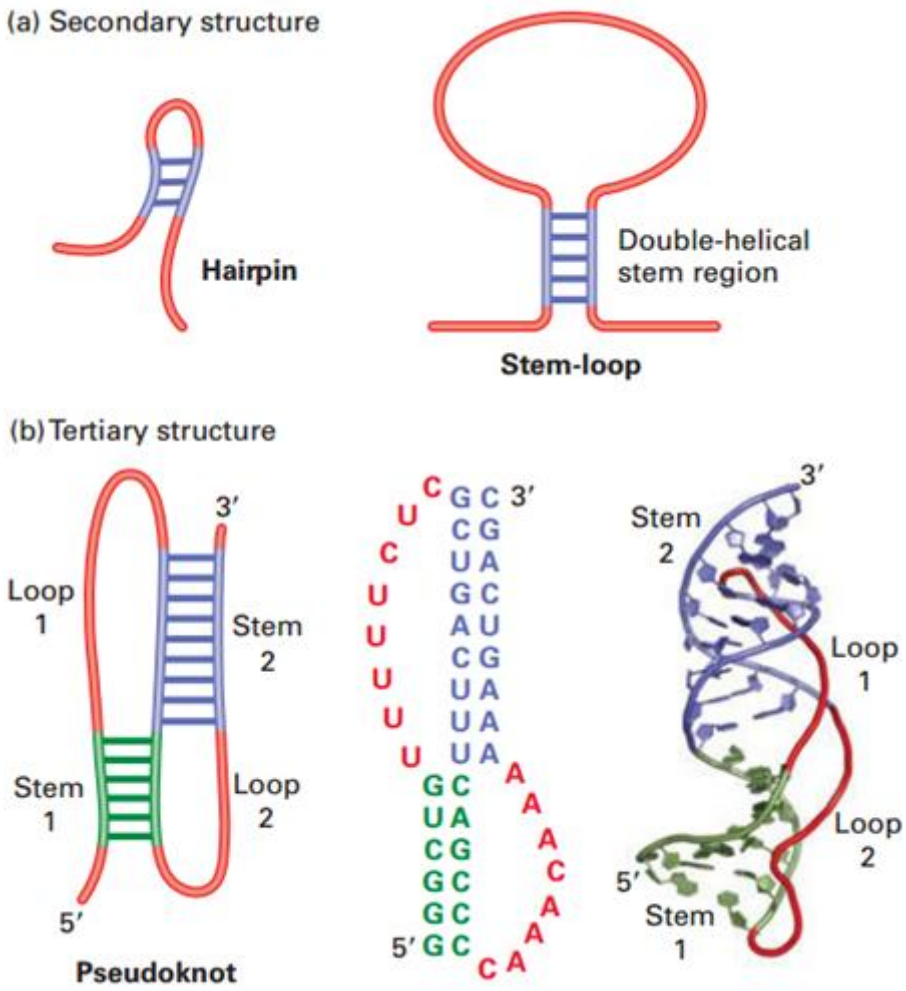

Fig. 5. RNA secondary and tertiary structures.

(a) Hairpins, stem-loops, and other secondary structures can form by base pairing between distant complementary segments of an RNA molecule. In stem-loops, the single-stranded loop between the base-paired helical stem may be hundreds or even thousands of nucleotides long, whereas in hairpins, the short turn may contain as few as four nucleotides. (b) Pseudoknots, one type of RNA tertiary structure, are formed by interaction of loops through base pairing between complementary bases. The structure shown forms the core domain of the human telomerase RNA. Left: Secondary-structure diagram with base-paired nucleotides in green and blue and single-stranded regions in red. Middle: Sequence of the telomerase RNA core domain, colored to correspond to the secondary-structure diagram at the left. Right: Diagram of the telomerase core domain structure determined by 2D-NMR, showing paired bases only and a tube for the sugar phosphate backbone, colored to correspond to the diagrams at left.

Key Concepts in DNA Replication

- Each strand in a parent duplex DNA acts as a template for synthesis of a daughter strand and remains base-paired to the new strand, forming a daughter duplex (semiconservative replication). New strands are formed in the 5′→3′ direction.
- Replication begins at a sequence called an origin. Each eukaryotic chromosomal DNA molecule contains multiple replication origins.

- DNA polymerases, unlike RNA polymerases, cannot unwind the strands of duplex DNA and cannot initiate synthesis of new strands complementary to the template strands.

- At a replication fork, one daughter strand (the leading strand) is elongated continuously. The other daughter strand (the lagging strand) is formed as a series of discontinuous Okazaki fragments from primers synthesized every 100 to 200 nucleotides.

- The ribonucleotides at the 5′ end of each Okazaki fragment are removed and replaced by elongation of the 3′ end of the next Okazaki fragment. Finally, adjacent Okazaki fragments are joined by DNA ligase.

- Helicases use energy from ATP hydrolysis to separate the parent (template) DNA strands, which are initially bound by multiple copies of a single-stranded DNA-binding protein, RPA. Primase synthesizes a short RNA primer, which remains base-paired to the template DNA. This primer is initially extended at the 3′ end by DNA polymerase α (Pol α), resulting in a short (5′)-RNA-(3′)DNA daughter strand.

- Most of the DNA in eukaryotic cells is synthesized by Pol δ and Pol ε, which take over from Pol α and continue elongation of the daughter strands in the 5′→3′ direction. Pol δ synthesizes most of the length of the lagging strand, while Pol ε synthesizes the leading strand. Pol δ and Pol ε remain stably associated with the template by binding to PCNA, a trimeric protein that encircles the daughter duplex DNA, functioning as a sliding clamp.

- DNA replication generally occurs by a bidirectional mechanism in which two replication forks form at an origin and move in opposite directions, and both template strands are copied at each fork.

- MCM helicases initiate eukaryotic DNA replication in vivo at multiple origins spaced along chromosomal DNA. Synthesis of eukaryotic DNA is regulated by controls on the binding and activity of these helicases.

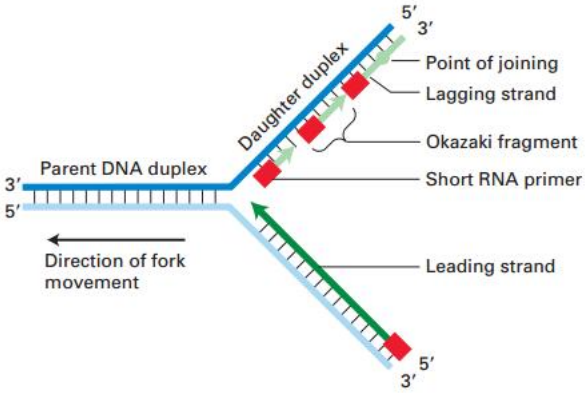

Fig. 6.Leading-strand and lagging-strand DNA synthesis.

Nucleotides are added by a DNA polymerase to each growing daughter strand in the 5′→3′ direction (indicated by arrowheads). The leading strand is synthesized continuously from a single RNA primer (red) at its 5′ end. The lagging strand is synthesized discontinuously frommultiple RNA primers that are formed periodically as each new region of the parent duplex is unwound. Elongation of these primers initially produces Okazaki fragments. As each growing fragment approaches the previous primer, that primer is removed and the fragments are ligated. Repetition of this process eventually results in synthesis of the entire lagging strand.

References:

1. Molecular Cell Biology – Harvey Lodish
2. Molecular Biology of Gene – James Watson
3. Molecular Biology of the cell – Bruce alberts
4. Cell and Molecular biology – Geraldkarp.

Polymerase Chain Reaction (PCR) – General Principles and Practices

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1. Introduction

Disease diagnosis is an important step in shrimp aquaculture system. The rapid, accurate and sensitive disease detection methods are need of the hour to evolve possible disease preventive measures. Several molecular methods, particularly Polymerase chain reaction (PCR) has become inevitable tool and being widely used for shrimp disease diagnosis. PCR is a highly sensitive and robust technique, derives its name from one of its key components, a DNA polymerase used to amplify a portion of DNA by *in vitro* enzymatic replication. The discovery of thermostable DNA polymerase from the thermophilic bacterium *Thermus aquaticus* led to rapid application of PCR in diagnostics. The PCR was first discovered by Kary Mullis during 1983 and was awarded Nobel Prize in Chemistry in 1993 for his contribution. The amplification is achieved using oligonucleotide primers that are specific for the portion of the DNA to be amplified. By designing oligonucleotide primers that are specific for an organism, it is possible to design PCR to amplify specifically DNA from any desired organism. As PCR progresses, the DNA thus generated is itself used as template for replication which is further exponentially amplified.

2. Equipment's and Materials

PCR machine,
Electrophoresis apparatus,
Adjustable micropipettes,
Micro-centrifuge tubes,
UV transilluminator,
Vortex mixer,
Spinner,
Adhesive tape,
Disposable gloves

3. PCR Components and reagents

All the chemicals and reagents should be of molecular biology grade. Sterilized condition should be well maintained in all operations as a preliminary requisite.

3.1. Buffer solution

It provides a suitable chemical environment for optimum activity and stability of the DNA polymerase.

3.2. Mg^{2+} concentration

The Mg^{2+} ion forms complex with dNTPs, primers and DNA template. It plays major role in the yield of PCR product. So the optimal concentration of MgCl2 has to be selected for each reaction. Too few Mg^{2+} ions result in a low yield of PCR product, and too many increase the yield of non-specific products, and promote misincorporation and multiple bands to appear. Lower Mg^{2+} concentrations are desirable when fidelity of DNA synthesis is critical. The concentration of MgCl2 should be selected empirically, starting from 1 mM and increasing in 0.1 mM steps, until a sufficient yield of PCR product is obtained. If the DNA samples contain EDTA or other chelators, the MgCl2 concentration in the reaction mixture should be raised proportionally.

3.3. dNTPs

The concentration of each dNTP in the reaction mixture is usually 200 µM. Inaccuracy in the concentration of even a single dNTP dramatically increases the misincorporation level. So it is very important to have equal concentrations of each dNTP (dATP, dCTP, dGTP, dTTP). When maximum fidelity of the PCR process is crucial, the final dNTP concentration should be 10–50 µM.

3.4. Primers

PCR primers are usually 17–30 nucleotides in length. Longer primers provide higher specificity. The CG content in primers should be 40 – 60% and distributed uniformly throughout of the primer. To avoid nonspecific priming, more than three G or C nucleotides at the 3'-end of the primer should be avoided. The primers should have neither self-complementarity nor inter-complementarity in order to avoid primer-dimer and hairpin formation. The melting temperature of flanking primers should not differ by more than 5°C, so the GC content and length must be chosen accordingly. The annealing temperature should be approximately 5°C lower than the melting temperature of primers.

3.5. DNA Polymerase

In 1957, Arthur Kornberg identified the first DNA polymerase and was awarded the Nobel Prize in 1959 for the same. In 1969, Thomas Brock reported the isolation of a new species of thermophilic bacterium, *Thermus aquaticus* which resides in the outflows of thermal pools in Yellowstone National Park from which Taq DNA polymerase was isolated in 1976. The enzyme, Taq DNA polymerase is worldover widely used in PCR reaction. It should be stored at –20°C in a frost free freezer, typically in 50% glycerol. The tubes should never be allowed to reach room temperature and gloves should be worn when handling to avoid contamination. Before opening a new tube of enzyme, it is spun briefly as there is often enzyme in the cap. When pipetting enzyme from a stock tube, the end of the tip is just plunged far enough into the enzyme to get what is needed to avoid excessive adherence of enzyme to the peripheral tips. Enzyme should never be added to unbuffered water to avoid its denaturation. Usually 0.5–1.0 U of *Taq* DNA polymerase is used in 50 µl of reaction mix. Higher *Taq* DNA polymerase concentrations may cause synthesis of nonspecific products.

3.6. Distilled Water

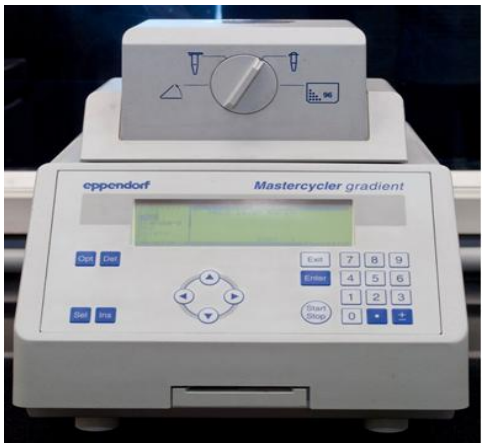
Autoclaved nuclease free distilled water is used to make up the desired volume for the reaction.

3.7. DNA template

It contains the DNA region (target) in question to be amplified.

4. The Cycling Reaction

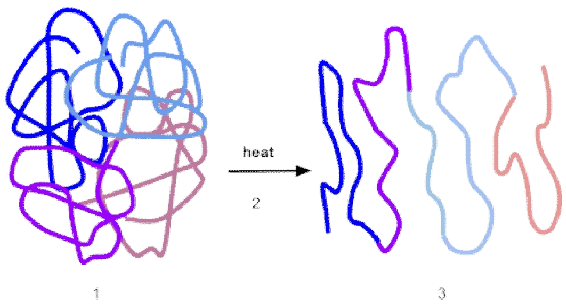
There are three major steps in a PCR, which are repeated for 30 to 40 cycles depending on the product size. This is done on an automated cycler, which can heat and cool the tubes with the reaction mixture in a very short time.



Thermal cycler

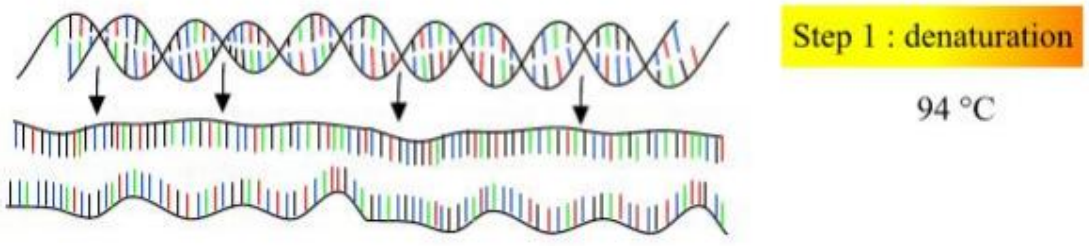
4.1. Initial Denaturation

The initial denaturation should be performed over an interval of 1–3 min at 95°C. This interval may be extended up to 10 min for GC-rich templates. If the initial denaturation is no longer than 3 min at 95°C, *Taq* DNA polymerase can be added into the initial reaction mixture. If longer initial denaturation or a higher temperature is necessary, *Taq* DNA polymerase should be added only after the initial denaturation, as the stability of the enzyme dramatically decreases at temperatures over 95°C.



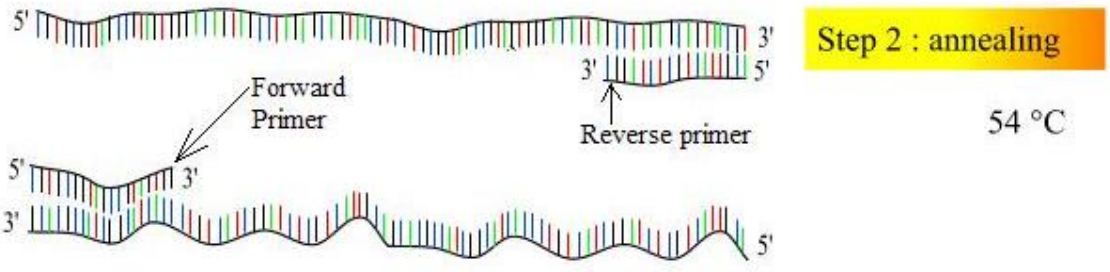
4.2. Denaturation

During the denaturation, the double strand melts open to single stranded DNA which is important in PCR reaction. Incomplete denaturation of DNA results in the inefficient utilization of template in the first amplification cycle leads to a poor yield of PCR product. Usually denaturation for 0.5–2 min at 94–95°C is sufficient. If the amplified DNA has a very high GC content, denaturation time may be increased up to 3–4 minutes. Alternatively, additives such as glycerol (up to 10–15 vol.%), DMSO (up to 10%) or formamide (up to 5%) may be used to facilitate DNA denaturation. In the presence of such additives, the annealing temperature should be adjusted experimentally, since the melting temperature of the primer-template DNA duplex decreases significantly when these additives are used. The amount of enzyme in the reaction mix should be increased since DMSO and formamide, at the suggested concentrations, inhibit *Taq* DNA polymerase by approximately 50%.



4.3. Annealing

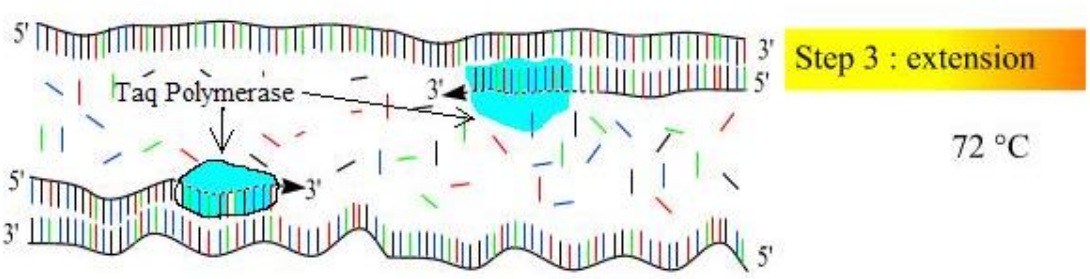
Usually the optimal annealing temperature is 5°C lower than the melting temperature of primer-template DNA duplex. Incubation for 0.5–2 min is usually sufficient. The annealing temperature should be optimized by increasing it stepwise by 1–2°C to avoid nonspecific PCR products. The primers are jiggling around, caused by the Brownian motion and ionic bonds are constantly formed and broken between the single stranded primer and the single stranded template. The more stable bonds last a little bit longer (primers that fit exactly) and on that little piece of double stranded DNA (template and primer), the polymerase can attach and starts copying the template. Once there are a few bases built in, the ionic bond is so strong between the template and the primer which does not break anymore.



4.4. Extension

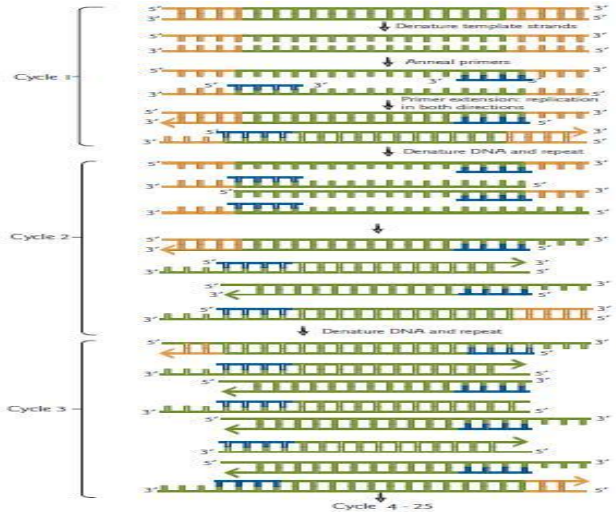
Usually the extension step is performed at 70–75°C. The rate of DNA synthesis by *Taq* DNA polymerase is highest at this temperature. Recommended extending time is 1 min for the synthesis of PCR fragments up to 2 kb and may be further increased by 1 min for each 1000 bp. Primers that are on positions with no exact match don't give an extension of the fragment. The polymerase adds complementary dNTP's to the template from 5' to 3', reading the template from 3' to 5'. Because both strands are copied during PCR, there is an exponential increase of the number of copies of the gene. But, polymerization is not strictly doubling the DNA at each cycle in the early phase. The number of PCR cycles depends on the amount of template DNA in the reaction mix and on

the expected yield of the PCR product. Usually 25–35 cycles are sufficient.



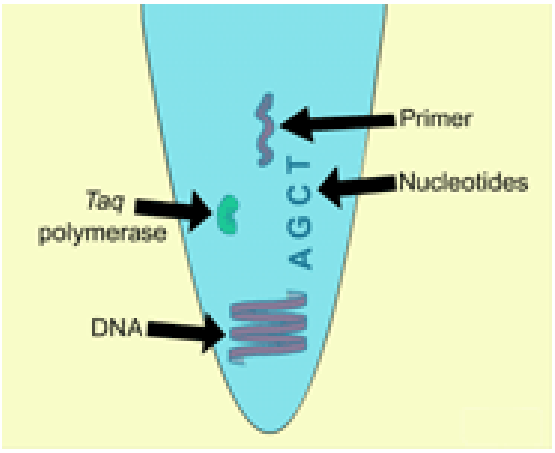
4.5. Final Extension

After the last cycle, the samples are usually incubated at 72°C for 5–15 min to fill-in the protruding ends of newly synthesized PCR products. Also, during this step, the terminal transferase activity of *Taq* DNA polymerase adds extra A nucleotides to the 3'-ends of PCR products.



5. PCR reaction set up

All the necessary reagents and enzymes needs to be added to a PCR tube. The amounts are calculated based on the total reaction volume. PCR enzymes and reagents are extremely temperature sensitive and therefore, care should be taken to keep it in ice or cooling box. It is preferable to prepare master mixes if several samples are there to analyze at the same time. For each PCR reaction, a positive control and a negative control are included.



PCR reaction components

An example for a typical reaction of 50µl set up:

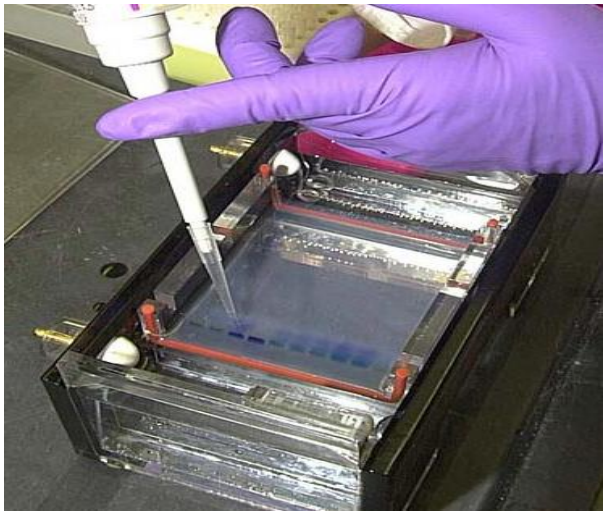
Buffer with $MgCl_2$ (10x)	: 5 µl
Primer F (10 pm)	: 1 µl
Primer R (10 pm)	: 1µl
dNTP (Mixture of 10mM each)	: 1µl
Taq (2.5 unit/µl)	: 0.5 µl
DNA	: 1– 2 µl
Water	: - µl (Make up to 50 µl)

The PCR can be standardized and optimized either by changing the denaturing time, annealing time and temperature, Mg^{2+} ion concentration, extension time and temperature, and or the individual quantity ingredients in reaction mixture. The final product is visualized in transillumination to know the amplification of the product of the interest.

6. Gel separation of PCR products

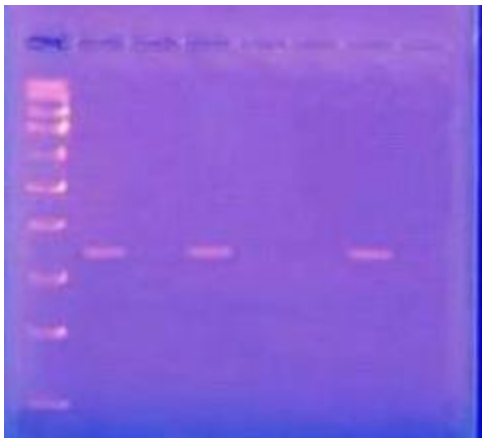
Based on the size of the amplified product, 0.8 to 2% agarose gels are prepared either in 1x Tris-Acetate-EDTA buffer (1 litre 50x TAE – 242 g Tris

base, 55 ml Glacial acetic acid and 37.2 g EDTA, pH 8) or 0.5 x Tris Boric acid EDTA buffer (1 litre 50x TBE – Tris base 540 g, Boric acid 275 g and EDTA 18.5g, pH 8.0). Ethidium bromide is added to the molten agarose (0.5 µg/ml final concentration) and then poured into the base. Once the gels are solidified, it is submerged in the tank with the same buffer. The amplified products are then mixed with 6x gel loading dye (For 100 ml – 30 mg Bromo Phenol Blue, 30 mg Xylene cyanol, 12 ml of 0.5M EDTA pH8, 1ml of 1M Tris-Hcl pH8, 27 ml of distilled water and 60 ml of sterile glycerol). A total volume of 5 to 10 µl is added to each well. A molecular weight marker is also loaded to the gel to verify the size of the amplified product. After loading, the tank is connected to a power pack and electrophoresis is carried out at voltage of 80 – 120 V. Continue the electrophoresis until the dye migrates to the appropriate distance in the gel.



7. Observation and documentation

The gel is finally put in a gel-doc for complete analysis or on a UV-transilluminator for visualization. The positive result is read in the form of a band at the right position in the gel. Absence of band indicates negative reaction or absence of virus. Presence of band in the positive control and absence of band in the negative control indicates absence of technical error or contamination.



PCR products visualised under UV-transilluminator

8. Record maintenance

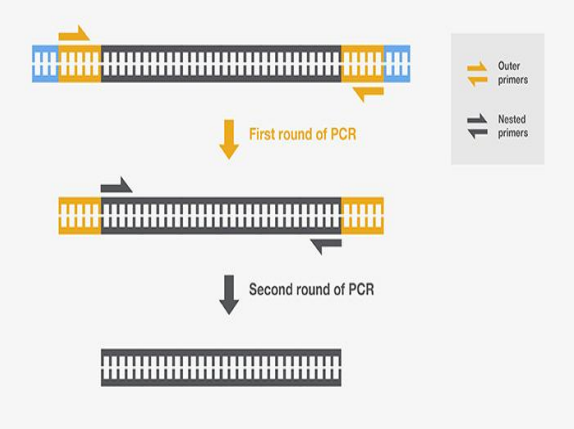
It is necessary to maintain a record regarding the results of each sampling. This will help to interpret the overall situation over a period of time.

9. Variants of PCR technique (Nested, RT PCR, qPCR, Multiplex)

The methods are being modified to make it more sensitive and more rapid. While direct PCR can detect comparatively higher load of pathogens (moderate to advance stage of infection), nested PCR can even detect the presence of very low number of pathogens in the host (initial stage of infection or asymptomatic carriers).

9.1. Nested PCR

The sensitivity and specificity are the most important parameters of a detection method; nested PCR has been developed for this purpose, in which two sets of PCR primers are sequentially used. The first primer set amplifies a target sequence, which then serves as the template for a second amplification. The second primer set lies internal to the first amplicon. This secondary amplification will not occur if the primary amplification did not happen. A major shortcoming of nested PCR is that the reaction vessel needs to be opened to add the second primer set which increases the contamination probability from the laboratory environments.



9.2. Reverse-transcription PCR

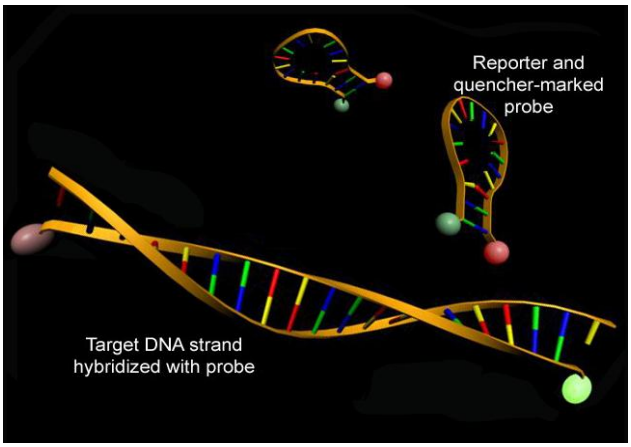
In reverse-transcription PCR, the RNA target as of a RNA virus is first converted into a complementary DNA (cDNA) by the reverse transcriptase enzyme. This cDNA is used as template and amplified by standard PCR methods. Reverse-transcription PCR is used not only to detect pathogens, but also to detect the specific expression of certain genes during the course of growth or infection since they are amplified at a much higher number of messenger or ribosomal RNA than the number of DNA copies. In contrast to the detection of DNA from nonviable organisms using standard PCR, the detection of cDNA from messenger RNA encoded by a pathogen using reverse-transcription PCR could be evidence of active infection.

9.3. Real-time PCR

Real-time PCR which is used to amplify and simultaneously quantify a targeted DNA molecule enables detection and quantification of the viral pathogen in the tissues of infected shrimp. It offers continuous monitoring of PCR product formation throughout the reaction and eliminates post-PCR analysis process. Thus, it shortens detection time compared to standard PCR, and reduces the risk of amplicon contamination by frequent handling during various steps of conventional PCR. By using this technique the viral load in infected shrimp can be accurately determined which in turn helps in risk assessment as well as disease monitoring during culture.

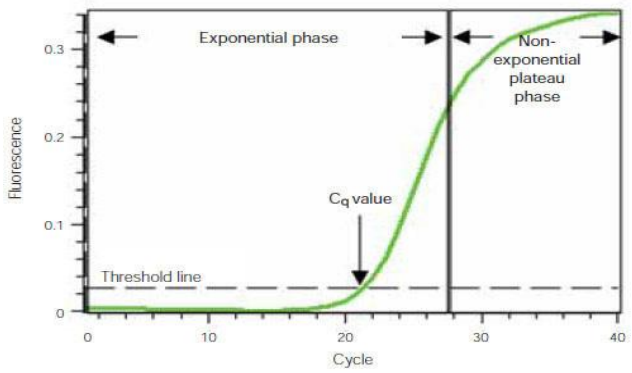
Four types of indicators have been used most frequently in real-time PCR methods for pathogen detection: TaqMan probes, SYBR Green dyes, molecular beacons, fluorescence resonance energy transfer (FRET) hybridization probes. SYBR Green chemistry is a method used to perform real-time PCR analysis. SYBR Green is a dye that binds to the minor groove of double stranded DNA. Here the intensity of the fluorescence emission increases with the amount of SYBR Green dye that binds to double stranded DNA. As the synthesis of double stranded amplicons continues in an exponential manner, SYBR Green dye signal increases.

In TaqMan probe, a single stranded oligonucleotide probe complementary to a segment of 20 to 60 nucleotides with in DNA template and located between the two primers is used. In this assay a fluorescent reporter and quencher are covalently attached to the 5’ and 3’ ends of the probe, respectively. The single stranded probe does not show fluorescence due to close proximity of fluorochrome and quencher. During PCR the 5’ to 3’ exonuclease activity of *Taq* polymerase degrades the portion of the probe that has annealed to the template, releasing the fluorochrome from proximity to the quencher. Thus fluorescence is directly proportional to the fluorophore released and amount of DNA template present in the PCR product.



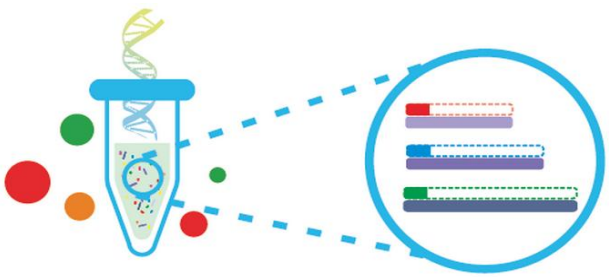
In real time PCR assay, the exponential increase in the fluorescence is used to determine the cycle threshold (Ct), which is the number of PCR cycles at which significant exponential increase in fluorescence is detected. Using a standard curve for

Ct values at different DNA concentrations, quantitation of target DNA in any sample can be made. Real time PCR assays have been successively applied for detection and quantification of IHHNV, TSV, WSSV, YHV, HPV etc. The real time multiplex PCR for the detection of more than two viral pathogens has also been developed.



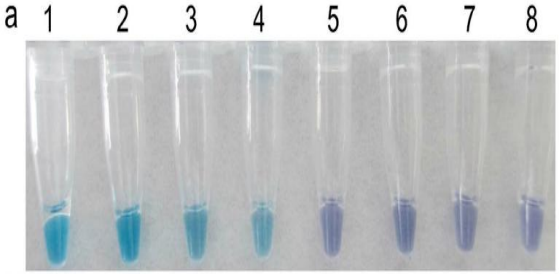
9.4. Multiplex PCR

Multiplex PCR has been successfully applied in many areas of nucleic acid diagnostics. The cost and limited volume of test samples are the key points for the pathogen detection. The process is termed multiplex PCR, since multiple sets of primers are included in a single reaction tube. In this procedure, more than one target sequence is amplified in a single reaction system by including more than one pair of primers. A key point in the development of a multiplex PCR assay is the design of the primers. All of the primers must be designed with very close annealing temperature, and the amplification products need to be of markedly different sizes so as to be easily differentiated by agarose gel electrophoresis. In addition, the multiplex primers might cause interference in the amplification process, which often makes it difficult for optimization of the reaction, especially when the number of primer pairs in the reaction system increases.

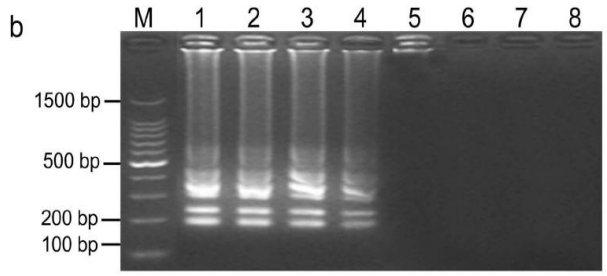


9.5. Loop-Mediated Isothermal Amplification (LAMP)

Loop-mediated isothermal amplification (LAMP) is a nucleic acid amplification method using single temperature incubation. It allows amplification of DNA with high specificity, sensitivity and rapidity. The amplification of nucleic acid is based on the principle of strand displacement DNA synthesis by the *Bst* DNA polymerase large fragment. The specificity, sensitivity and rapidity of LAMP are due to the high strand displacement activity of the *Bst* polymerase and a set of two inner primers and two outer primers.This technique can amplify target nucleic acid to 10^9 copies at 60–65 ^{0}C within 1 h. LAMP is highly specific for the target sequence because of the recognition of the target sequence by six independent sequences in the initial stage and by four independent sequences in the later stages of the LAMP reaction. The amount of amplicons generated can be quantified in real-time either by measuring the turbidity or by the signals produced by fluorescent dyes that intercalate the DNA. As the reaction is conducted under isothermal conditions, it can be carried out with a simple and inexpensive water bath so that a thermal cycler is not required. In addition to being inexpensive, isothermal amplification technique is further simplified by the use of chromatographic, lateral flow dipstick. Rapid detection of viruses by LAMP of genomic material with high specificity and sensitivity can be applied for diagnosis, monitoring and control of diseases in shrimp aquaculture. LAMP has been developed for the detection of major shrimp viruses including TSV, YHV, WSSV, IMNV, IHHNV, MBV, and HPV.



(a) LAMP and hydroxynaphthol blue (HNB) visualization



(b) LAMP and gel electrophoresis

9.6. PCR–Enzyme Linked Immunosorbent Assay (PCR–ELISA)

The PCR–ELISA is an alternative method for the detection of nucleic acids which mimic enzyme linked immunosorbent assays. The technique mainly involves amplification of viral DNA by PCR followed by hybridization of the PCR product with a specific probe and finally the detection of the hybridized product by ELISA technique. In this assay, the PCR products will be hybridized to an immobilized capture probe with sequences internal to the PCR product. Thus, it is an alternative and less expensive technique than real-time PCR. PCR–ELISA, a promising diagnostic tool has been developed for detection of major shrimp viruses. This technique could detect up to three viral particles. Hence, PCR–ELISA is more sensitive than conventional PCR and histological examination and can be used for field level applications where large numbers of samples can be analyzed simultaneously.

10.Probe Techniques

The development of non-radioactive labelling of nucleic acid fragments has made gene probe technology readily available in shrimp disease diagnosis. This technology was first developed for the diagnosis of IHHNV and now it is being used for other shrimp viruses. Non-radioactively labelled digoxigenin (DIG) DNA probe has been used in dot blot, *in situ* hybridization and southern blot hybridization for detection and analysis of major viral pathogens of *L. vannamei viz* IHHNV, TSV, YHV, WSSV, HPV, MBV etc.

11. Application of PCR

1. PCR is used in diagnostic application in pathology for the detection of infectious agents and the discrimination of non-pathogenic from pathogenic strains by virtue of specific sequence.
2. Different PCR-based methods are used in genetic fingerprinting to identify the extremely small amounts of target of interest.
3. Used to identify genetic and evolutionary relationships between living organisms.
4. Used to identify beneficial and pathogenic microbiota at strain level by techniques such as ribotyping, randomly amplified polymorphic DNA (RAPD), pulsed-field gel electrophoresis (PFGE) of rare-cutting restriction fragments, and amplified fragment length polymorphism (AFLP).
5. Recombinant DNA technologies involving the insertion of a DNA sequence into a plasmid or the genetic material of another organism.
6. PCR may also be used in the analysis of ancient DNA that is thousands of years old.
7. Larger quantities of DNA are required for Southern or northern hybridization and DNA cloning. PCR augments these techniques with high amounts of pure DNA, enabling analysis of DNA samples even from very small amounts of starting material.
8. DNA sequencing.

PCR is a useful tool for rapid identification of viral and other shrimp pathogens. PCR has been widely applied to the detection of shrimp viruses so that the risk of disease can be controlled. Early detection in larvae will help to discard the batch before taking it into culture practice. This includes screening of broodstock, larvae and post larvae in the hatchery before stocking. PCR is also used for identifying carriers, checking water and sediment for viral contamination and monitoring health of shrimp in grow out ponds. Detection in culture ponds will provide clue to take biosecurity measures to prevent the spreading.

12. PCR diagnosis of important OIE listed viruses and bacterial pathogens of shrimp

Table 1. Primer sequence for OIE listed aquatic pathogens of shrimp

Sl. No.	Pathogen	Primer Sequence 5' to 3'	Amplicon size	
			First step	Nested
1	WSSV , Kimura et al (1996)	F: ATCATGGCTGCTTCACAGAC R: GGCTGGAGAGGACAAGACAT	982 bp	570 bp
		Fn: TCTTCATCAGATGCTACTGC Rn: TAACGCTATCCAGTATCACG		
2	IHHNV , OIE, 2015	F:TCCAACACTTAGTCAAAACCAA R: TGTCTGCTACGATGATTATCCA	309 bp	-
3	MBV, OIE, 2015	F:CGATTCCATATCGGCCGAATA R:TTGGCATGCACTCCCTGAGAT	533 bp	361 bp
		Fn:TCCAATCGCGTCTGCGATACT Rn:CGCTAATGGGGCACAAGTCTC		
4	HPV, OIE, 2015	F:GCATTACAAGAGCCAAGCAG R:ACACTCAGCCTCTACCTTGT	441 bp	-
5	IMNV, OIE, 2015	Fn:CGACGCTGCTAACCATACAA Rn:ACTCGGCTGTTCGATCAAGT	328 bp	139 bp
		Fn:GGCACATGCTCAGAGACA Rn:AGCGCTGAGTCCAGTCTTG		
6	YHV, OIE, 2015	F:CCGCTAATTTCAAAAACTACG R:AAGGTGTTATGTCGAGGAAGT	135 bp	-
7	TSV, OIE, 2015	F:AAGTAGACAGCCGCGCTT R:TCAATGAGAGCTTGGTCC	231 bp	-
8	NHPB, OIE, 2015	F:CGTTGGAGGTTCGTCCTTCAGT R:GCCATGAGGACCTGACATCATC	379 bp	-
9	AHPND Dangtip et al., (2015)	F: ATGAGTAACAATATAAAACATGAAAC R: ACGATTTCGACGTTCCCCAA	1269bp	230bp
		Fn: TTGAGAATACGGGACGTGGG Rn: GTTAGTCATGTGAGCACCTTC		
10	EHP Tangprasittipap et al. (2013)	F:CAGCAGGCGCGAAAATTGTCCA R:AAGAGATATTGTATTGCGCTTGCTG	779 bp	176 bp
		Fn:CAACGCGGGAAAACTTACCA Rn:ACCTGTTATTGCCTTCTCCCTCC		

F: Forward primer R: Reverse primer Fn: Nested Forward primer Rn: Nested Reverse primer

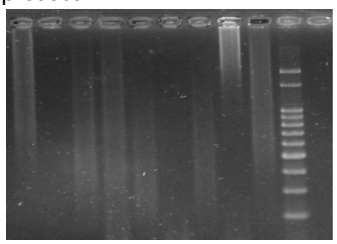
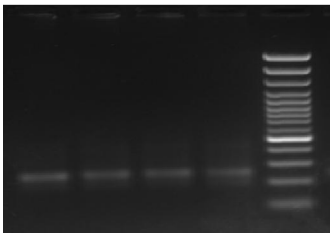
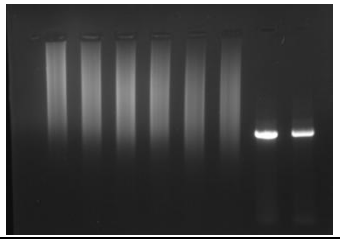
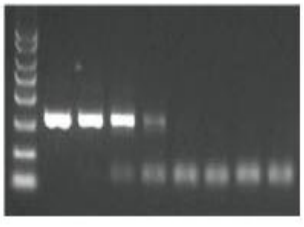
Thermocycling

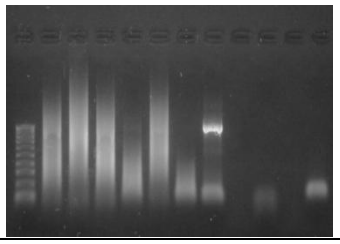
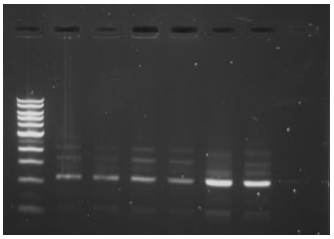
The tubes are to be arranged in the thermal cycler. Care should be taken to close it properly to avoid evaporation. Cyclic conditions for the amplification of some important pathogens of shrimp are listed in Table given below:

Table 2. PCR condition forpathogens of shrimp

Sl. No.	Pathogen	PCR protocol
1	WSSV , Kimura et al (1996)	Cycle 1:94^{0}C for 5 min Cycle 2:94^{0}C for 40sec 58^{0}C for 40sec 72^{0}C for 90sec Go to cycle 2 repeat 30 cycles Cycle 3:72^{0}C for 7 min
2	IHHNV , OIE, 2014	Cycle 1: 95^{0}C for 5 min Cycle 2:95^{0}C for 30sec 55^{0}C for 30sec 72^{0}C for 1 min Go to cycle 2 repeat 35 cycles Cycle 3: 72^{0}C for 7 min
3	MBV, OIE, 2014	Cycle 1: 96^{0}C for 5 min Cycle 2:94^{0}C for 30sec 65^{0}C for 30sec 72ºC for 1 min Go to cycle 2 repeat 40 cycles Cycle 3: 72^{0}C for 7 min
4	HPV, OIE, 2014	Cycle 1: 95ºC for 5 min Cycle 2:95^{0}C for 30sec 60^{0}C for 30sec 72^{0}C for 60sec Go to cycle 2 repeat 40 cycles Cycle 3: 72^{0}C for 7 min
5	IMNV, OIE, 2014	Cycle 1: 60^{0}C for 30 min 95^{0}C for 2 min Cycle 2:95^{0}C 45 sec 60^{0}C 45 sec Go to cycle 2 repeat 39 cycles Cycle 3: 60^{0}C for 7 min
6	YHV, OIE, 2014	Cycle 1: 94^{0}C for 2 min Cycle 2:94^{0}C for 30sec 58^{0}C for 30 sec 72^{0}C for 30sec Go to cycle 2 repeat 40cycles Cycle 3: 72^{0}C for 10min
7	TSV, OIE, 2014	Cycle 1:94^{0}C for 2 min Cycle 2:94^{0}C for 45 sec 60^{0}C for 45sec 72^{0}C for 45sec Go to cycle 2 repeat 40cycles Cycle 3:72^{0}C for 7 min
8	NHPB, OIE, 2014	Cycle 1:95^{0}C for 2min Cycle 2:94^{0}C for 30sec 60^{0}C for 30sec 72^{0}C for 30sec Go to cycle 2 repeat 25cycle Cycle 3:72^{0}C for 7min
9	AHPND Sirikharin et. al, (2014)	Cycle 1: 94^{0}c for 5min Cycle 2:94^{0}C for 30sec 53^{0}C for 30sec 72^{0}C for 40sec Go to cycle 2 repeat 35 cycles Cycle 3:72^{0}C for 5 min
10	EHP Tangprasittipap et al. (2013)	Cycle 1: 94^{0}C for 5min Cycle 2: 94^{0}C for 20sec 58^{0}C for 20sec 72^{0}C for 45sec Go to cycle 2 repeat 35 cycles Cycle 3:72^{0}C for 5min
		Nested Cycle 1: 94^{0}C for 5min Cycle 2: 94^{0}C for 20sec 64^{0}C for 20sec 72^{0}C for 45sec Go to cycle 2 repeat 35cycles Cycle 3:72^{0}C for 5min

13. Trouble shootings in PCR

Observation	Possible Cause	Possible solution
No PCR product 	Insufficient number of PCR cycles	Replace the PCR vials and run an extra 5 cycles
	DNA-template degenerated	Check DNA quality by electrophoresis
	Thermocycler programme is not correct	Check temperatures and cycle time
	Inhibitors present, which slow down the PCR	Reduce the volume of sample in the reaction mix; carry out another ethanol precipitation with the samples
	Missing reaction component	Check the reaction components and carry out a new PCR
	Suboptimal reaction conditions	Optimize Mg2+ concentration, annealing temperature and elongation time. Always vortex PCR buffer. Primers should be present in equal concentrations
	PCR vials not autoclaved	Autoclaving PCR vials prevents contamination, while inhibitors can notinterfere with the reaction
	Nucleotides degenerated	Store nucleotides in frozen batches, thaw quickly and keep on ice. Prevent frequent freeze/thaw cycles
	Error in gel analysis.	Check that the gel was loaded correctly and stained properly
Amplification with low yield. 	Enzyme activation not long enough (or too long) or the temperature not high enough	Check manufacturer's recommendations
	Annealing temperature too high or too low.	Optimise the annealing temperature using a gradient if possible.
	Annealing or extension time too short.	Increase the hold times. For long products (>2kb) use incremented hold times on the extension step.
	Not enough cycles	Increase the number of cycles
	Not enough template.	Increase the amount of template
No template control (NTC) shows amplification. 	Amplicon or template contamination of one of the reagents.	Repeat the assay with fresh reagents. Separate PCR set up from analysis. Use filter tips
Non-specific products: primer dimers 	Short non-specific products amplified in preference to the target.	Reduce primer concentration; reduce MgCl2 concentration; use a hot start enzyme; use a touchdown PCR protocol; re-design primers

Observation	Possible Cause	Possible solution
Non-specific products: smeared bands on gel 	Degraded template and/or reactions conditions too permissive.	Check template integrity. Increase stringency of reaction – use a touchdown PCR method
	Long products not amplified completely.	Increase extension time – use an incremented hold time on the step. Ensure the mix contains enough reagents such as dNTPs.
	Primers not specific	Check primer specificity
Non-specific products : Multiple bands 	Premature replication	Use a hot start polymerase, such as OneTaq Hot Start DNA Polymerase Set up reactions on ice using chilled components and add samples to thermocycler preheated to the denaturation temperature
	Primer annealing temperature too low	Increase annealing temperature
	Incorrect Mg++ concentration	Adjust Mg++ in 0.2–1 mM increments
	Contamination with exogenous DNA	Use positive displacement pipettes or non-aerosol tips Set-up dedicated work area and pipettefor reaction setup Wear gloves during reaction setup
	Incorrect template concentration	For low complexity templates (i.e. plasmid, lambda, BAC DNA), use 1 pg–10 ng of DNA per 50 µl reaction For higher complexity templates (i.e. genomic DNA), use 1 ng–1 µg of DNA per 50 µl reaction
	Primer concentration high	Check primer concentration and decrease concentration, if necessary

References

1. Innis MA, DH Gelfand, JJ Sninsky, and TJ White (eds.) 1990 PCR Protocols: A Guide to Methods and Applications. San Diego, California: Academic Press.
2. McPherson MJ, SG Moller, R Beynon, and C Howe 2000 PCR: Basics from Background to Bench. Heidelberg: Springer-Verlag
3. M. Joshi and J. DeshpandePolymerase chain reaction: methods, principles and application (2011).International Journal of Biomedical Research, 2, pp. 81–97
4. M.T. Rahman, M.S. Uddin, R. Sultana, A. Moue, M. Setu Polymerase chain reaction (PCR): a short review . Anwer Khan Modern Med. College J., 4 (2013), pp. 30–36.

Collection, preservation and processing of shrimp samples for detection of pathogens by PCR

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Introduction

For investigating disease, moribund sample (infected animals those are about to die) are best samples for diagnosis. However, individuals from the same pond may also be collected fixed appropriately and provided to the diagnostic laboratory. Collection, storage and archiving of specimens and tissue samples are prerequisites for the successful acquisition of molecular data for any systematic study. This chapter reviews the important practical aspects of the sampling and storage: 1) selection of appropriate tissues for nucleic acid extraction 2) storage of freshly collected tissues in the field 3) transportation, long-term storage and archiving of tissue samples.The likelihood that this study will yield statistically significant results depends on the sample size. Sample sizes are directly dependent on the assumptions on the number of animals present in the pond or tank (table). Sample numbers should be adequate to understand the prevalence of disease at a given confidence limit

Sample sizes needed to detect at least one infected host in a population of a given size, at a given prevalence of infection.

Population size	Prevalence (%)						
	0.5	1.0	2.0	3.0	4.0	5.0	10.0
50	46	46	46	37	37	29	20
100	93	93	76	61	50	43	23
250	192	156	110	75	62	49	25
500	314	223	127	88	67	54	26
1000	448	256	136	92	69	55	27
2500	512	279	142	95	71	56	27
5000	562	288	145	96	71	57	27
10000	579	292	146	96	72	57	27
100000	594	296	147	97	72	57	27
1000000	596	297	147	97	72	57	27
>10000000	600	300	150	100	75	60	30

Sampling method for disease diagnosis

Sampling and fixation is one of the most crucial steps for precise shrimp disease diagnosis. Sampling must ensure an accurate representation of the health status of the population or individual. Sampling can be either lethal or nonlethal. Non-lethal sampling is usually carried out with brood stocks, where tip of the pleopod or faecal threads are used for PCR without sacrificing the animal. For lethal sampling, entire larvae or any tissue material such as hemolymph, gill, muscle, pleopod, lymphoid organ, hepatopancreas and eye stalk collected based on the type of viral pathogen needed for detection. Egg and larvae (~ 150 numbers of egg or larvae- nauplii to mysis) or ~10 PL depending on size/age) can be taken as whole and pooled sample to represent a mass. In case of late PL, it is preferred to cut the head and take abdominal portion to avoid PCR inhibitors. 3-6 diseased or moribund shrimps and an equal number of normal shrimps should be collected and packed separately.

The selection of particular tissue type is mandatory in the accurate diagnosis of viral infection. Because shrimp viral pathpgen infects particular cells and tissues of a host which support growth of a particular virus. Some viruses have a broad tissue tropism and can infect many types of cells and tissues. Other viruses may infect primarily a single tissue. For example White spot syndrome virus (WSSV) infects ectodermal and mesodermal origin tissues such as epidermis, gills, pleopod and hemolymph. But monodon baculovirus(MBV) infects only endodermal origin tissue hepatpancreas. The selected tissue of the organism should be relatively free of compounds potentially damaging to the nucleic acid or interfere with PCR. For example, Eye balls are known to contain PCR inhibitors.

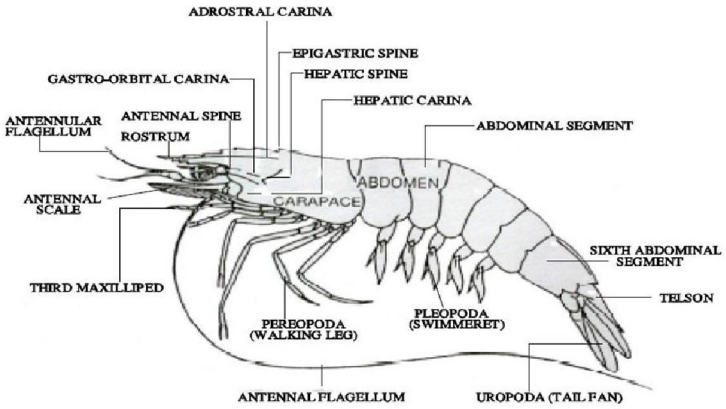

Fig1: Morphology of Shrimp

Target organs of DNA, RNA viruses, bacteria and Parasites infecting shrimp

DNA Viruses	Abbreviation	Target organs	Genome
White spot syndrome virus	WSSV	larvae, Pleopod, gill, hypodermis , hemocytes	dsDNA
Monodon baculovirus	MBV	larvae, hepatopancreas	dsDNA
Baculoviral midgut gland necrosis virus	BMNV	larvae, hepatopancreas	dsDNA
Baculovirus penaei	BP	larvae, hepatopancreas, anterior midgut	dsDNA
Spawner isolated mortality virus	SMV	larvae, hepatopancreas,midgut	ssDNA
Hepatopancreatic parvovirus	HPV	larvae, hepatopancreas	ssDNA
Infectious hypodermal haeamtopoietic necrosis virus	IHHNV	larvae,Pleopod, gill, hypodermis, haematopoietic tissues, lymphoid organ	ssDNA
RNA Viruses	Abbreviation	Target organs	Genome
Yellow head virus	YHV	larvae, gill, gut, gonads, pleopod,hemocytes, lympoid organ	(+)ssRNA
Taura syndrome virus	TSV	larvae, gill, gut, striated muscle, pleopod, hypodermis, lympoid organ	(+)ssRNA
Infectious myonecrosis virus	IMNV	larvae, Skletal muscles, lympoid organ, hemocytes	(+)ssRNA
Macrobrachium rosenbergii nodavirus	MrNV	larvae, gill,pleopod, muscle, ovary	(+)ssRNA
Mouriliyan virus	MoV	larvae, gill, lympoid organ, cuticular epithelium	(-) ssRNA
Gill associated virus	GAV	larvae, gill, gut, gonads, pleopod,hemocytes, lympoid organ	(+)ssRNA
Lymphoid organ vacuolization virus	LOVV	larvae, lympoid organ,	(+)ssRNA
Laem singh virus	LSNV	larvae, gills, lympoid organ, nervous tissues	dsRNA
PARASITE	Abbreviation	Target organs	Genome
Enterocytozoon hepatopenaei	EHP	larvae, Hepatopancreas	ds DNA
Bacteria	Abbreviation	Target organs	Genome
Necrotizing hepatopancreatitis	NHP	larvae, Hepatopancreas	ds DNA
Acute hepatopancreatic necrosis disease	AHPND	larvae, Hepatopancreas, bacterial culture from gut tissue of live shrimp	ds DNA

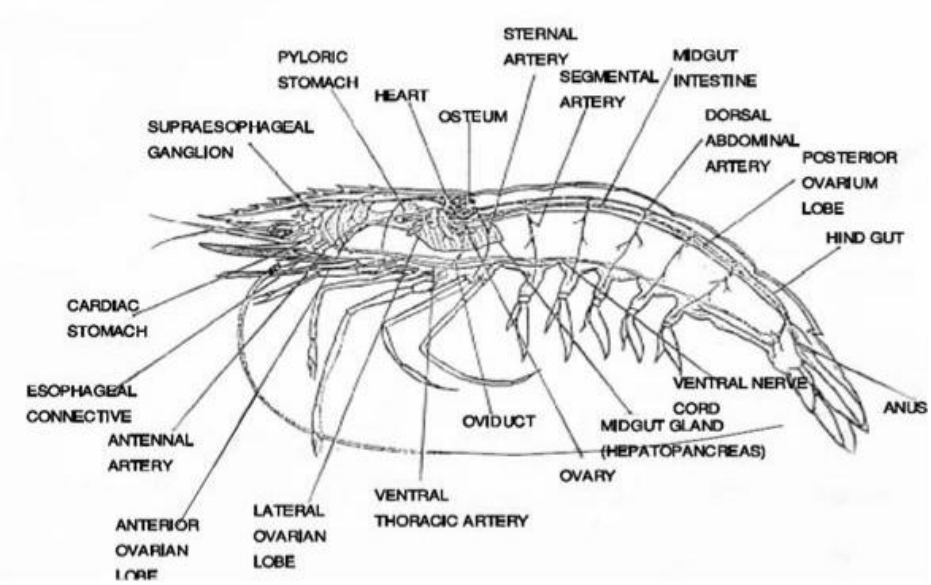

Fig 2: Anatomy of shrimp

Egg or larvae ~ 150 nos

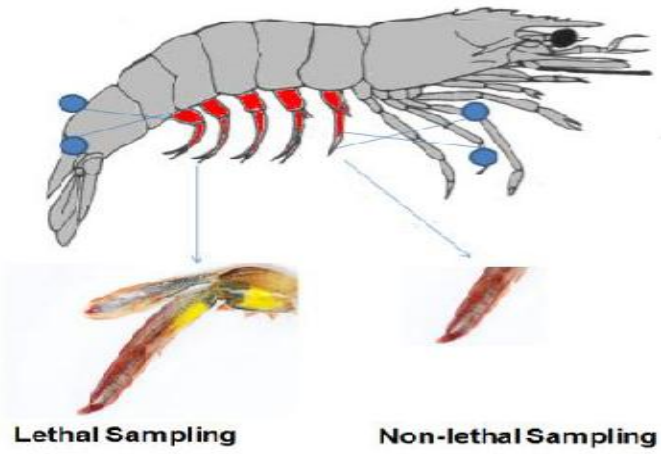

Fig 3: Sampling of shrimp for disease diagnosis

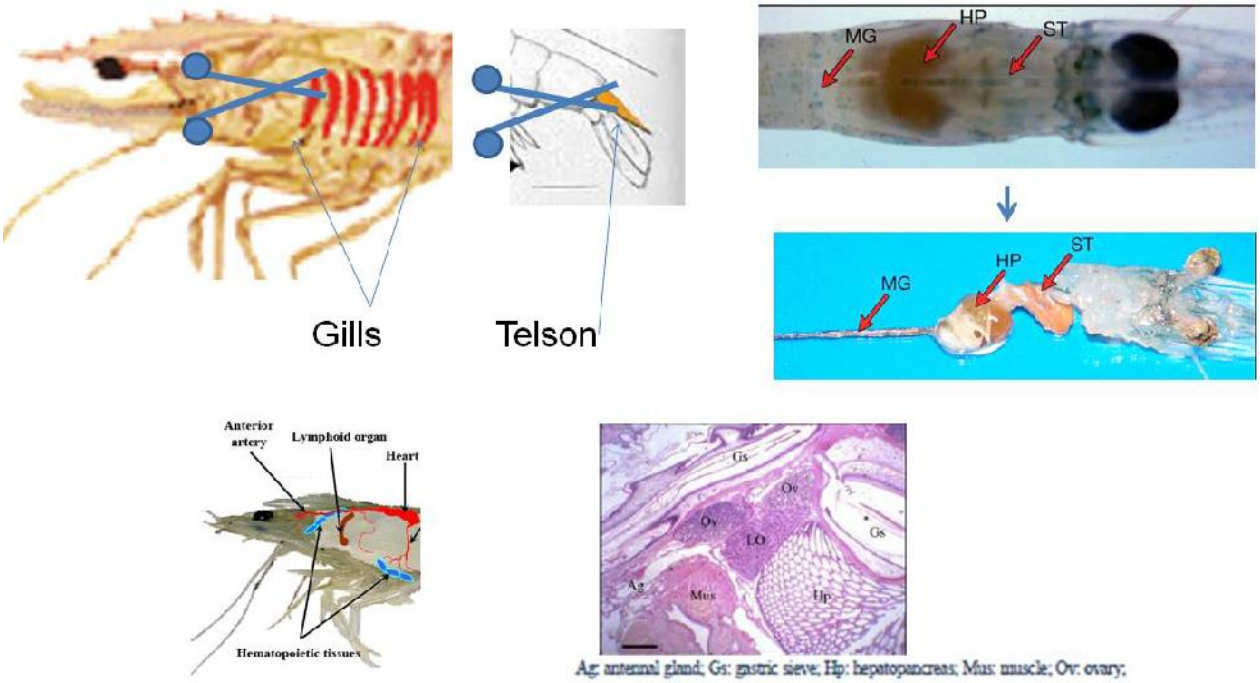

Fig 4: Tissues of diagnostic importance

Preservation of tissue

Fresh material from live animals consistently provides the highest yield and quality of nucleic acidfor amplification. The live animals or moribund animals can be frozen in dry ice and rapidly placed in the cold and away from light. The tissues should be packed in plastic cryotubes or Ziploc bags excluding as much air as possible to avoid cross contamination.

The tissue samples can be stored and transported in 95–100% ethanol at ambient temperature. The larger size or exoskeleton of the animal does not allow the penetration of ethanol of the tissue and causes degradation of the tissues. These samples should be injected with ethanol, dissected into smaller pieces to allow the ethanol to diffuse directly into the internal tissues. There should be about ten volumes of ethanol to one volume of sample for the proper preservation of the sample. Ethanol should be replaced after the initial fixation and periodically at a regular interval.

Long-term storage conditions should minimise variation in temperature. The animal tissues will remain indefinitely stable for extraction of nucleic acids at –70–80 °C.

This will allows the archiving of samples for reanalysis. There are also several commercial preservative available specifically to preserve nucleic acid in tissue.

Steps to avoid Contamination

The investigator should be aware of the importance of keeping their instruments, containers and reagents clean in order to prevent cross-contamination. The individual tissue samples should be stored in separate containers. The investigator should label and document all materials they collect with the details such as date of collection, collector, voucher number, etc with the permanent ink markers.

Extraction of Nucleic Acid from Shrimp Tissue

Introduction

The extraction of DNA and RNA is the most crucial method used in molecular biology. These biomolecules can be isolated from any biological material for the diagnosis of pathogenic virus, bacteria and protozoa using PCR. In the past, the process of extraction and purification of nucleic acids used to be complicated, time-consuming, labour-intensive. Currently, there are many

specialized methods that can be used to extract nucleic acids, such as solution-based and column-based protocols. Automated systems designed for medium-to-large laboratories have grown in demand over recent years. It is an alternative to labor-intensive manual methods.

Principle

Several components have been used in the extraction of nucleic acid. The role various components of DNA extraction protocol is as follows:

A. The extraction buffer: It includes a detergent such as cetyl trimethyl ammonium bromide(CTAB) or SDS which disrupts the membranes, a reducing agent such as B mercaptoethanol which helps in denaturing proteins by breaking the disulfide bonds between the cysteine residues, a chelating agent such as EDTA which chelates the magnesium ions required for DNAse activity and a salt such as sodium chloride which aids in precipitation by neutralizing the negative charges on the DNA so that the molecules can come together.

B. Phenol chloroform extraction: Nucleic acid solutions commonly contain undesirable contaminants that are chiefly made of proteins. A classic method of purifying is phenol –chloroform extraction by which the contaminants are denatured and and accumulate in the organic phase or in the marginal layer between the two phases and the nucleic acids are preserved in the aqueous phase. The proteins can also be removed by using the enzyme proteinase K which however again is denatured by phenol via phenol chloroform extraction.

C. Precipitation of nucleic acids: Alcohol precipitation is the most commonly used method for nucleic acid precipitation. The nucleic acid was diluted with a monovalent salt and precipitated by the addition of alcohol to it with gentle mixing. The precipitated nucleic acid can be pelleted by centrifugation. The salts and alcohol remnants are removed by washing with 70% alcohol. The most commonly used salts include sodium acetate pH 5.2 (final concentration 0.3M), sodium chloride (final concentration 0.2M). Ethanol (twice the volume) or isopropanol (two thirds volume) are the standard alcohols used for nucleic acid precipitation.

D. Resuspending DNA: The nucleic acid pellet can be resuspended in either sterile distilled water or TE(10 mM Tris:1mM EDTA)

E. Purification of DNA: The DNA is purified by incubating the nucleic acid solution with RNAse A (10mg/ml) at 37° C and reprecipitation following phenol: chloroform extraction to remove the RNase.

Equipments and Materials

Refrigerated centrifuge, Deep freezer, Ice flaker, Shaking water bath, Sphectrophotometer, Vortex mixer, Autoclaved Milli Q water, Adjustable mocropipettes,Micro-centrifuge tubes,Homogenizer, Disposable gloves.

Sample material: Specific tissue from shrimp or bacterial colony for DNA isolation.

Method I : DNA extraction by lysis method

Composition of Lysis buffer

- 10mM Tris Hcl pH 8.0
- 25mM EDTA
- 0.5% SDS
- 100mM NaCl
- Make up to 100ml with dd water.
- Add 0.5 µl proteinase K (20mg/ml) to the lysis buffer just before use to make the working concentration of proteinase K @10 µg/ml

1. Take 20 to 50 mg tissue (PL, pleopod, gill, or 50 ul haemolymph sample) in a 1.5ml microcentrifuge tube. The frozen tissue should be thawed directly in lysis buffer, ethanol preserved tissue should be blotted dry with fresh tissue and add the tissue to a 1.5 ml microcentrifuge tube containing 150 µl lysisbuffer and homogenate gentlyand completely withthe disposable pestle and then add 350 µl lysis buffer.

2. Incubate the tissue homogenate at 95°C for 10 min with intermittent mixing. Allow the homogenate to cool down to room temperature.
3. Centrifuge at 12000g for 10 minutes at 4° C and transfer 200 µl of the upper clear solution to a fresh 1.5ml tube with 400 µl ethanol.
4. Centrifuge again at 12000g for 5 minutes at 4° C and carefully discard the supernatant and air dry the pellet.
5. Dissolve the pellet by sterile milliQ water or TE buffer.

Method II: DNA extraction by CTAB method

Reagents

1. Extraction(CTAB) Buffer: 4 M Sodium chloride, 100 mM Tris (pH 8.0), 20 mM EDTA (pH 8.0),-2% β-Mercaptoethanol,2% CTAB
2. Isopropanol
3. Saturated phenol pH 8.0
4. Chloroform : isoamylalcohol (24:1) mixture
5. Tris:EDTA (10mM:1mM) pH 8.0
6. RNase A (10mg / ml): Dissolve RNase A in 10mM Tris-Cl, pH 7.5, 15 mM NaCl. Heat at 100°C for 15 min. Cool to room temperature and store as aliquots at -20° C.
7. 70% ethanol

Procedure

1. Take 25 to 100mg tissue (PL, pleopod, gill, or 50 ul haemolymph sample) in a 1.5ml microcentrifuge tube. The frozen tissue should be thawed directly in CTAB buffer, ethanol preserved tissue should be blotted dry with fresh tissue and add the tissue to a 1.5 ml microcentrifuge tube containing 150 µl CTAB buffer and homogenate gentlyand completely with the disposable pestle and then add 800 µl buffer.
2. Incubate the tissue homogenate at 65°C for about one hour with intermittent mixing. Allow the homogenate to cool down to room temperature.
3. Vortex briefly the homogenate and then add 0.7 ml of chloroform, vortex for another 20 seconds, and then centrifuge at 12000 gfor 5 minutes.
4. Transfer 700 µl upper aqueous phase to a new 1.5 microcentrifuge tube.
5. Add 630 µl (0/9 volume) isopropanol and mix gently and allow the DNA to precipitate for 30 min.by keeping it in – 20° C deep freezer.
6. Centrifuge at 12000g for 5 minutes, then wash the pellet with 600 ul of 70% ethanol.
7. Centrifuge again at 12000g for 5 minutes at 4° C and carefully discard the supernatant and air dry the pellet.
8. Re-suspend in TE buffer.
9. Add RNAse A and incubate at 37° C for one hour.
10. Add equal volume of phenol : chloroform : isoamyl alcohol (25:24:1), mix properly forat least 5 min and centrifuge at 12000 gfor 10 minutes. Extract twice with chloroform : isoamyl alcohol.
11. Precipitate the DNA by adding 1/10 volume of 3M sodium acetateand 2 volume of ice cold ethanol. Mix gently and spool out the DNA or precipitate by centrifugation at 12,000 g for 10 minutes.
12. Remove extra salts by washing the pellet with 70% ethanol. Air dry the pellet and resuspend the pellet with TE buffer and store at – 20° C.

Isolation of RNA using TRIZOL reagent:

Reagents

- TRIZOL® Reagent
- Chloroform
- Isopropyl alcohol
- 75% Ethanol (in DEPC-treated water)
- RNAse-free water or 0.5% SDS solution

DEPC inactivates the RNases by the covalent modifications of the histidine residues. To prepare RNase-free water, draw water into RNAse-free glass bottles. Add diethylpyrocarbonate (DEPC) to 0.01% (v/v). Let stand overnight and autoclave.

Tissue Homogenization

Homogenize the tissue samples with 1 ml of TRIZOL reagent per 50 to 100 mg of tissue using a disposable sterile Teflon homogenizer. The sample volume should not exceed 10% of the volume of TRIZOL Reagent used for the homogenization. Incubate the homogenized sample for 5 minutes at room temperature to permit the complete dissociation of nucleoprotein complexes. Centrifuge to remove cell debris. Transfer the supernatant to new tube.

Phase separation

Add 0.2 ml of chloroform per 1 ml of TRIZOL Reagent. Cap sample tubes securely. Vortex samples vigorously for 15 seconds and incubate them at room temperature for 2 to 3 minutes. Centrifuge the samples at no more than 12,000 x g for 15 minutes at 2 to 4°C. Following centrifugation, the mixture separates into lower red, phenol chloroform phase, an interphase, and a colorless upper aqueous phase. RNA remains exclusively in the aqueous phase. Transfer upper aqueous phase carefully without disturbing the interphase into fresh tube. Measure the volume of the aqueous phase (The volume of the aqueous phase is about 60% of the volume of TRIZOL Reagent used for homogenization).

RNA precipitation

Precipitate the RNA from the aqueous phase by mixing with isopropyl alcohol. Use 0.5 ml of isopropyl alcohol per 1 ml of TRIZOL Reagent used for the initial homogenization. Incubate samples at 15 to 30°Cfor 10 minutes and centrifuge at not more than 12,000 x g for 10 minutes at 2 to 4°C. The RNA precipitate, often invisible before centrifugation, forms a gel-like pellet on the side and bottom of the tube. G: RNA WASH: Remove the supernatant completely. Wash the RNA pellet once with 75% ethanol, adding at least 1 ml of 75% ethanol per 1 ml of TRIZOL Reagent used for the initial homogenization. Mix the samples by vortexing and centrifuge at no more than 7,500 x g for 5 minutes at 2 to 8 oC. Repeat above washing procedure once. Remove all leftover ethanol.

Redissolving RNA

Air-dry or vacuum dry RNA pellet for 5-10 minutes. Do not dry the RNA pellet by centrifuge under vacuum. It is important not to let the RNA pellet dry completely as this will greatly decrease its solubility. Partially dissolved RNA samples have an A260/A280 ratio < 1.6. Dissolve RNA in DEPC-treated water by passing solution a few times through a pipette tip.

Fractionation of nucleic acid by agarose gel electrophoresis

Agarose Electrophoresis

Agarose gel electrophoresis is a method of gel electrophoresis used in molecular biologyto separate nucleic acids based on their size and charge. These gels are easy to cast and are widely used in laboratories. An agarose is a polysaccharide polymer material, generally extracted from seaweed. It is a linear polymer made up of the repeating unit of agarobiose, which is a disaccharide made up of D-galactose and 3,6-anhydro-L-galactopyranose.The melting temperature of agarose is 85-95 °C and gelling temperature of 35-42 °C. The nucleic acids have a net negative charge due to its phosphate back bone so they migrate towards the positive electrode in an electric field. The migration of nucleic acids affected by several factors like pore size of the gel, size of DNA being electrophoresed, the voltage used the ionic strength of the buffer, and the concentration of intercalating dye such as ethidium bromide.

1. Agarose gel preparation

a. To prepare a gel required quantities of agarose weighed and add in to the wide mouth glass conical flask with 1x TAE buffer and melt the mixture in the microwave

oven, until it becomes clear without any gel particle.

b. Cool down the clear molten agarose gel under room temperature and add 1 µl ethidium bromide (10mg/ml) and slowly pour the gel into the gel mould without air bubble. The volume of the gel varies from the size of the gel mould. The height of agarose gel only has to go above the bottom of the gel comb for about 0.3~0.5 cm, and thickness is suggested to be no less than 0.8 cm.

c. When agarose gel is completely solidified. Carefully remove blockers at both sides of the gel mould and place it in the gel tank containing 1x TAE buffer.After few minutes the comb will loosen up in the gel and can be carefully removed without damaging the wells. This agarose gel is ready for electrophoresis.

2. Electrophoresis

a. Add 1X TAE buffer over the gel box until the buffer level submerge the gel.

b. Load 5 µl each of the "PCR product-loading dye mixture" into each well. The mixture will sink to the bottom of the wells because its density is higher than buffer. This step should be carefully handled in order to avoid cross contamination between the adjacent wells.

c. DNA marker is loaded at the extreme end of the gel. The DNA molecular weight marker is served as reference to predict the size of the PCR product.

e. After loading of all the samples, the gel is electrophoresed at constant voltage between 70V~100V.

e. The loading dye Bromphenol Blue gives deep blue color; Xylene Cyanol gives light blue color. When the dark blue dye approaches 1/2 to 2/3 of the gel, stop the electrophoresis. Then, remove the gel from the gel mould to observe under UV light.

3.Staining and visualization

The ethidium bromide intercalates into the major grooves of the DNA and fluoresces under UV light. So the gel can be viewed under transilluminator (254nm) to observe DNA bands. The exposure of DNA to UV radiation for as little as 45 seconds can produce damage to DNA and affect subsequent procedures such as cloning. The exposure of the DNA to UV radiation therefore should be limited. The use of a higher wavelength of 365 nm UV light causes lesser damage to the DNA.The transilluminator apparatus fitted with image capture devices, such as a digital or polaroid camera allow an image of the gel to be stored in a computer or printed.

Detection of White Spot Syndrome Virus (WSSV) byOIE PCR protocol

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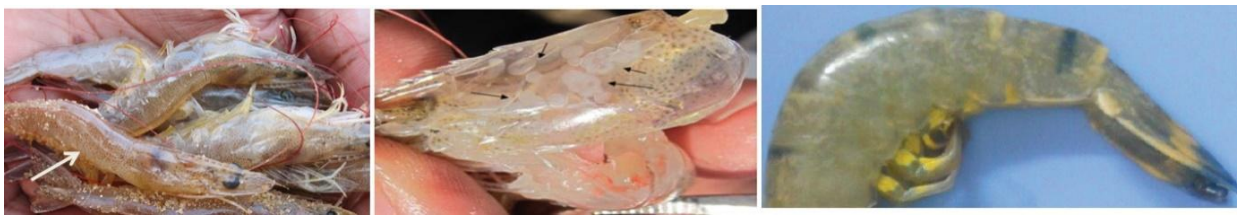
White spot is one of the most serious diseases of shrimp which has been reported throughout the world. It causes severe economic losses through mass mortality and repeated crop failure to shrimp farming industry. Originating from China during 1992-93, this disease later spread to different parts of the world. The majority of crustaceans including all the penaeid shrimps, some species of crabs and the freshwater craw fish are considered as major hosts. Many of the other crustaceans, some molluscs and annelids are known to be carriers and help in disease spread. Towards late 1994, this disease was already present in India and responsible for mass mortality in many of the shrimp farms. Even after two decades of prevalence the disease is still causing high level of mortality. All the life stages of shrimp are known to get infected by the virus. However, disease severrity and mortallity are recorded in juveniles and later stages after stocking in ponds. There has been no treatment methods for WSD and manintainance of BMP and stringent biosecurity can only prevent the occurrence.

Clinical signs

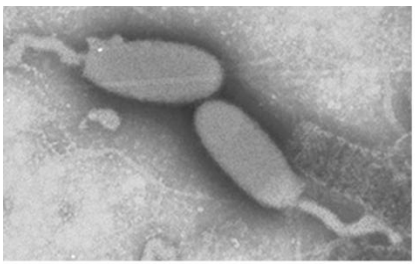
Shrimps with WSD exhibit anorexia and come to pond sides or surface. They become very lethargic and thereby easily picked up by predatory birds or crabs which becomes a means for disease spread. Many times they also show pink discoloration of the body. Affected shrimps start to cease feeding with the progress of the disease. Mortality starts within 3-4 days of disease onset and mass mortality may occur within 7-10 days.

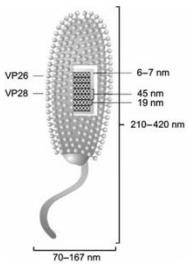


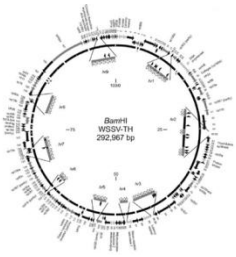
The disease is typically characterised by the presence of circular white spots on the carapace and throughout the abdomen. In some cases and particularly in vannmei, the white spots may not be that obvious to recognise the disease with the preliminary clinical sign.

Causative agent

The WSD is caused by a virus. This is a rod shaped virus with or without a tail.







The virus has ~ 300 kb double stranded DNA inside an envelope, and is the biggest known animal virus with several genotypes. The virus belongs to a new family, Nimaviridae and entirely a new genus, *Whispovirus*. While passing through different investigational stages, the virus has been named in

several ways as WSSV, SEMBV, RV-Pj, HHNBV, Pm NOB III etc.

WSD diagnosis by PCR

There have been several well standardised methods for the detection of WSSV. This ranges from simple direct microscopy to histopathology, *in situ* hybridization, PCR and LAMP. However, amongst all, PCR is the more popular as it is a rapid and sensitive technique for accurate and easy detection of the pathogen.

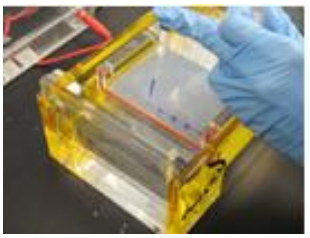
For the OIE protocol of detection of WSSV, following primers are used

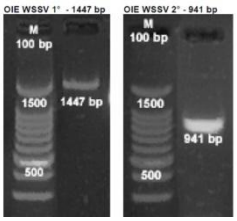
SL No.	Primer name	Primer Sequence	1st step/ Nested/ Control	Amplified product Size
1	146 F1	5'-ACT-ACT-AAC-TTC-AGC-CTA-TCTAG-3'	1st step Amplification	1447 bp
2	146 R1	5'-TAA-TGC-GGG-TGT-AAT-GTT-CTT-ACG-A-3'		
3	146 F2	5'-GTA-ACTGCC-CCT-TCC-ATC-TCC-A-3'	2nd Step/ Nested Product	941 bp
4	146 R2	5'-TAC-GGC-AGC-TGC-TGC-ACC-TTG-T-3'		
5	143 F	5'-TGC-CTT-ATC-AGCTNT-CGA-TTG-TAG-3'	Control/ Decapod specific primer	848 bp
6	145 R	5'- TTC-AGN-TTT-GCA-ACC-ATA-CTT-CCC-3 (N represents G, A, T or C)		

Once the reaction mix is ready, it is feed to a thermocycler. Cycling conditions include an initial denaturation step, denaturation, annealing, extension and a final extension consisting of 30-40 cycles. For OIE protocol, following are the cycling conditions;94°C for 4 minutes, 55°C for 1 minute, and 72°C for 2 minutes, followed by 39 cycles of 94°C for 1 minute, 55°C for 1 minute, and 72°C for 2 minutes and a final 5-minute extension at 72°C. A total of 35 cycles are run to complete the reaction.

Detection of amplified product

The amplified product is detected in an agarose gel (1% or so depending on product size in 1X TAE or 0.5X TBE) incorporated with ethidium bromide (0.5 µg/ml). The amplified products (5 to 10µl) are mixed with 6× gel loading buffer and added to individual wells of the gel. Many of the readily available master mix already contain the gel loading dye and therefore it can be directly added to the wells. One molecular weight marker is loaded as a standard to know the amplified product size. The gel is then run in either 1X TAE or 0.5 X TBE for about 1 hour by observing the dye movement.





The gel is then put either on a gel documentation system or UV-transilluminator for detection of bands. The software present in the gel documentation system can directly analyse the gel and capture the image. In case of the

transilluminator, the image has to be captured manually.

Interpretation of result

If amplified product is matched with the molecular weight marker and if the size is accurate, the sample is considered as positive. At the same time all the controls should also provide desired result. In case of any ambiguity, the product should be verified through sequencing. Similarly, if PCR is performed on a new species, the product should also get verified by sequencing. Once standardised and then practice routinely, in such cases, sequencing the product each time is not necessary. A print of the image should be taken and put on the record book with all other additional details

Detection of White Spot Syndrome Virus (WSSV) by PCR using IQ 2000 kit

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1.0 Introduction

WSSV was assigned by the International Committee on Taxonomy of Viruses (ICTV) as the only member of the genus Whispovirus within the Nimaviridae family. Virions of WSSV are ovoid or ellipsoid to bacilliform in shape, have a regular symmetry, and measure 80–120 nm in diameter and 250–380 nm in length. Most notable is the thread- or flagella-like extension (appendage) at one end of the virion. The agent is viable for at least 30 days at 30°C in seawater under laboratory conditions and is viable in ponds for at least 3–4 days.WSSV has an extremely wide host range. The virus can infect a wide range of aquatic crustaceans especially decapod, including marine, brackish and freshwater prawns, crabs, crayfish and lobsters.

All life stages are potentially susceptible, from eggs to broodstock. The best life stages of crustaceans for detection are late PL stages, juveniles and adults. Probability of detection can be increased by exposure to stressful conditions like, eye-stalk ablation, spawning, moulting, changes in salinity, temperature or pH, and during plankton blooms.The infection can be transmitted vertically (trans-ovum), horizontally by consumption of infected tissue (e.g. cannibalism, predation, etc.), and by water-borne routes. Transmission of infection can occur from apparently healthy animals in the absence of disease. Dead and moribund animals can be a source of disease transmission.

2.0 Sample Preparation

2.1 Requirements

- Micro Centrifuge tubes
- 95 % Ethanol
- Forceps, Scalpel, Scissors & Blade
- Sodium Hypo chloride (5%)

2.2. Processing Shrimp for Nucleic Acid Extraction

This section provides sample dissection procedures for different life cycle of Shrimp. After sampling is carried out follow the Nucleic acid extraction protocol as given in following sections. If samples preserved in Ethanol, dry completely before nucleic acid extraction.

2.2.1 Brood stock eye stalk

- Use the whole eye stalk and put into a 1.5 ml micro centrifuge tube.

2.2.2 Larvae, PL or Juvenile

- Place about 50 mg specimen into a 1.5 ml micro centrifuge tube. The test requires at least 50 pieces for larvae; 30 pieces for <PL12; for >PL12 use 30 tail pieces, only half tail should be used.

2.2.3 Pleopod & gill of Adult shrimp

- Place a piece of Pleopod/gill (50 mg) into a 1.5 ml micro centrifuge tube.

2.2.4 Tail muscle of Adult shrimp

- Place tail muscle (50 mg) into a 1.5 ml micro centrifuge tube.

2.2.5 Shrimp Egg &Nauplii

- Place 20 – 30 mg Egg or Nauplii sample into a 1.5 ml micro centrifuge tube.

3.0 DNA Extraction

3.1 Equipment required

- Dry bath
- Centrifuge
- Vortex Mixer
- Laminar air flow

3.2 Materials & Reagents required

- 1.5 ml Micro centrifuge tubes
- Dodecyl trimethyl ammonium Bromide (DTAB)
- Cetyl trimethyl ammonium bromide (CTAB)
- 95 % Ethanol &75 % Ethanol
- Chloroform
- Diethyl pyro carbonate (DEPC)treated Water
- Micropipette and Micro tips (ART)

3.3 DNA Extraction

3.3.1 IQ 2000 DTAB-CTAB DNA Extraction

- Add sample into a 1.5 ml tube with 600 µl DTAB solution and homogenise the sample with grinding sticks
- Incubate the prepared sample at 75^{O} C for 5 min, then cool down to room temperature (25^{0}C)
- Vortex briefly and spin down the mixture, then add 700 µl of chloroform, vortex for another 20 seconds and centrifuge at 12000 rpm for 5min.
- Add 100 µl of CTAB solution and 900 µl of Distilled water to a new tube and transfer supernatant to that tube.
- Vortex briefly, then incubate at 75 ^{O}C for 5 min
- Cool down to room temperature and centrifuge at 12000 rpmfor 10 min
- Carefully decant the supernatant, re-suspend the pellet with 150 µl Dissolving solution and vortex briefly.
- Incubate at 75^{O} C for 5 min and cool down to room temperature
- Spin at 12000 rpm for 5 min. Transfer the supernatant to a fresh 1.5 ml tube with 300 µl of 95% ethanol
- Vortex briefly, centrifuge at 12000 g for 5 min, then wash the pellet with 200 µl of 75 % ethanol, spin down, dry the pellet and dissolve in distilled water or DEPC water.

3.3.2 IQ 2000 Lysis Buffer DNA Extraction

- Add 500 µl Lysis Buffer in a 1.5ml tube.
- Transfer shrimp sample into the tube and homogenise the sample with grinding sticks.
- Incubate the prepared sample at 95°C for 10 minutes, then centrifuge at 12000 rpm for 10 minutes.
- Transfer 200 µl of the supernatant to a fresh 1.5ml tube with 400 µl 95% ethanol.
- Vortex briefly and centrifuge at 12000 rpm for 5 minutes then discard the ethanol and dry the pellet.
- Dissolve the pellet by DEPC water or TE buffer.

4.0 Dissolution of DNA

4.1 Materials required

- DEPC Water
- Micro centrifuge tubes
- Pipettes and Micro tips (ART)

4.2 Dissolution

A. The yield of DNA is different for different samples; therefore the concentration of DNA should be adjusted by dissolving the DNA pellet in appropriate volume of DEPC water.

Sample Source	Volume
Eye stalk of brood stock	100 µl
Post Larvae (PL)	200 µl
Pleopod or Periopod	200 µl
Gill	100 µl
Tissue / Muscle	300 µl

B. Adjust the volume of DEPC water according to the quality and quantity of the individual samples measured by using the spectrophotometer.

C. These DNA samples could be stored at– 20 ^{o}C.

4.3 Quality and Quantity measurement of Extracted DNA

4.3.1 Equipment required

- Spectrophotometer
- Vortex Mixer

4.3.2 Materials required

- Cuvette
- Pipettes and Micro tips (ART)
- DEPC Water

Quality and quantity of DNA is measured using spectrophotometer. Approximately 50 µg/ml dsDNA with the absorption ratio of 260/280 nm and the range is 1.7 to 2.0.

5.0 Amplification of DNA

5.1 Equipment required

- Thermo cycler
- PCR Workstation
- Vortex mixer
- Spin down centrifuge

5.2 Materials & Reagents requirement

- IQ2000 PCR Kit for WSSV
- 0.2 ml PCR tubes
- PCR Cooler racks for 1.5 ml micro centrifuge tubes and 96-well 0.2ml PCR reaction plates
- Sterile nuclease-free 1.5 ml& 0.2 ml micro centrifuge tubes
- Positive Controls & Negative Controls

5.3 Amplification Procedure

Standard PCR Protocol

A. First PCR Reaction reagent mixture: 8 µl reaction

First PCR Premix	7.5 µl
IQZyme DNA Polymerase (2U/µl)	0.5 µl

B. Nested PCR reaction reagent mixture: 15µl/reaction

Nested PCR Premix	14 µl
IQzyme DNA Polymerase (2U/µl)	1 µl

5.4 Reaction Procedure

- Prepare first PCR reaction reagents as per the no. of samples and standards to be tested.
- Pipette 8 µl of PCR reaction reagent mixture into each tube with proper label.
- Add 2 µl of the extracted sample DNA and standard into each tube with reaction mixture. Perform first PCR reaction.
- Prepare nested PCR reagent mix as per the no. of samples to be tested.
- Add 15 µl of nested PCR reaction reagent mixture to each tube after first PCR was completed. Perform nested PCR reaction.

5.5 Reaction condition

Premix (First Step PCR)

Reaction step	Temp	Time	Cycles
Initial Denaturation	94°C	2 min	1
Denaturation	94°C	20 sec	15
Annealing	62°C	20 sec	
Extension	72°C	30 sec	
Final Extension	72°C	30 sec	1
Hold	20°C	30 sec	1

Nested Mix

Reaction step	Temp	Time	Cycles
Initial Denaturation	94°C	20 sec	30
Annealing	62°C	20 sec	
Extension	72°C	30 sec	
Final Extension	72°C	30 sec	1
Hold	20°C	30 sec	1

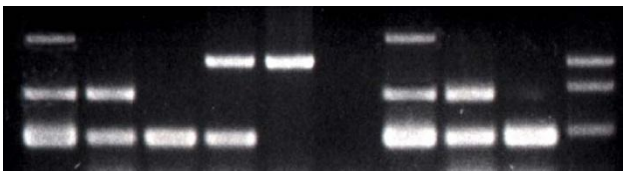
6.0 Electrophoresis

- Prepare 10 X TBE or TAE and dilute to 1X concentration.
- Prepare 2% agarose gel in a conical flask.
- Cool down the clear Agarose gel under room temperature about 25^0C.
- Add 2 µl of 10 mg/ml Ethidium Bromide for 100 ml of agarose gel and mix completely then slowly pour the gel into the casting tray. Allow the gel to set/solidify.
- The height of Agarose gel should only above the bottom of the gel comb for about 0.3~0.5 cm.
- Load 10~15µl of the PCR product with loading dye into each well.
- Add 5µl DNA markers for every batch. The DNA molecular weight marker provides a reference for the DNA size and position.
- After sample loading, connect the gel box with power supply before switching on. Adjust the voltage to 60~100 V.
- The 6X loading dye contains 2 colorants: Bromophenol Blue (deep blue colour) and Xylene Cyanole FF (light blue colour). Switch off the power supply when the deep blue colorant approaches 1/2 to 2/3 of the gel.
- Record the results and label the image using Gel Documentation System.
- Place the gels into 5% bleach solution overnight before disacard.

7.0 Diagnosis

Results of Samples and Controls / standards will show the following patterns on gel

1 23456789M



Lane 1: Sample of severe WSSV infection
Lane 2: Sample of moderate WSSV infection
Lane 3: Sample of light WSSV infection
Lane 4: Sample of very light WSSV infection
Lane 5: WSSV negative sample
Lane 6: Negative control (ddH_2O)
Lane 7: WSSV P (+) standard, 2000 copies/reaction
Lane 8: WSSV P(+) standard, 200 copies/reaction
Lane 9: WSSV P(+) standard, 20 copies/reaction
Lane M:Molecular weight marker, 848 bp, 630 bp, 333 bp

2. Negative samples will show only one band at 848 bp, which is a PCR product of housekeeping gene as internal control.
3. Diagnostic procedure:

a. Band formed at 296 bp and/or 550 bp: Postive

b. Band formed only at 848 bp: Negative

Detection of Infectious hypodermal hematopoietic necrosis (IHHNV) in shrimp Using OIE PCR protocol

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Introduction

Infectious hypodermal hematopoietic necrosis (IHHN) infection which is silently creating havoc among shrimp farmers seen distributed throughout the world causing severe viral epizootics affecting both commercial as well as wild penaeid populations.

Etiology

Infectious hypodermal and hematopoietic necrosis virus (IHHNV) recently referred as infectious *Penaeus stylirostris* densovirus (PstDNV) from the genus *Brevidensovirus* and in the *family Parvoviridae*causing IHHN infection in penaeids is one of the smallest known shrimp pathogen which causes negative impact on the shrimp farming economy. Since 1983 this disease remains as one of the OIE notifiable. This virus has three genotypes, Type 1 from Americas and East Asia, Type 2 from South – East Asia and both these types are infectious to penaeids while Type 3 found to be non- infectious to *P. vannamei* and/or *P. monodon* and was from East Africa, India, Australia and the western Indo-Pacific region.

Susceptible hosts

Several penaeid species are susceptible to the virus but, the severity and mortality rate of infection often differs between species, with *Penaeus stylirostris* to be most susceptible. All life stages of *P. vannamei*(i.e. eggs, larvae, post larvae, juveniles and adults) are susceptible.

Clinical signs

This disease caused high mortalities in young and old juveniles of *Penaeus stylirostris*. However, in *P. vannamei* a chronic form of disease is encountered in young and old juveniles resulting in a condition referred as Runt Deformity Syndrome (RDS) wherein the affected shrimp display a bent (45° to 90° bend to left or right) or otherwise deformed rostrum, a deformed sixth abdominal segment, wrinkled antennal flagella, cuticular roughness, 'bubble-heads', and other cuticular deformities. The affected farm show severe incongruentgrowth in size and shape of shrimp.

Microscopic pathology

Prominent intranuclear, Cowdry type A inclusion bodies which are eosinophilic and often haloed, characteristic to this disease aides in provisional diagnosis of IHHNV infection.

Disease transmission

Transmission occurs by both routes like vertical as well as by horizontal route (cannibalism and contaminated route)

Polymerase chain reaction method for IHHNV detection

Several methods like single step PCR, nested PCR are available to diagnose IHHNV infection. The OIE protocol is the most commonly used PCR protocol for the detection of IHHNV disease. Several primers are used for the detection of infectious and non-infectious forms of IHHNV. The infectious form of IHHNV can be diagnosed by using the primer set 309 F/R which amplifies segments only from IHHNV types 1 and 2 but not types 3A and 3B, which are non-infectious and part of the *P. monodon* genome (Tang & Lightner, 2006; Tang *et al.,* 2007). Two primer sets 392 F/R and 389 F/R are the most appropriate for diagnosing all the known genetic variants of IHHNV (Krabsetsve *et al.,* 2004; Tang & Lightner, 2002). They are capable of detecting IHHNV-related sequences called types 3A and 3B, which are inserted into the genome of certain geographical stocks of *P. monodon* from the

western Indo-Pacific, East Africa, Australia and India (Duda & Palumbi, 1999; Tang & Lightner, 2006; Tang *et al.,* 2007; Saksmerprome *et al.,* 2011). The non-infectious part of the *P. monodon* genome can be detected by using the primer sets MG831 F/R which reacts only with types 3A and 3B (Tang *et al.,* 2007).

Sampling

The principal target organs includes the gills, cuticular epithelium/ hypodermis, all connective tissues, the haematopoietic tissues, the lymphoid organ, antennal gland, haemocytes, ventral nerve cord, its branches and its ganglia and parenchymal cells. So it is recommended to collect the whole shrimp or tissue samples containing the target tissues which are suitable for molecular methods.

Pooling of samples

Diagnosis for juveniles, sub-adults and adults can be done by five specimens per pooled sample. However, for eggs, larvae and PL, larger numbers (e.g. ~150 or more eggs or larvae or 50–150 PL depending on their size/age) may be obligatory to obtain sufficient sample material (extracted nucleic acid) to run a molecular assay.

DNA extraction

The DNA can be extracted from the above said target tissues by alkaline lysis method or any other DNA extraction method as described in earlier chapter of this manual. It is always advisable to quantify the extracted DNA to know its purity before proceeding to the PCR reactions.

Preparations of PCR reaction mix

The PCR reactions can be performed either in 25 µl or 50 µl reactions. The reaction mixture for 25 µl should be of the following:

Reagents	Vol(µl)	Final concentration
Water	9.5	-
2X master mix (Buffer with $MgCl_2$, dNTPs, Taq DNA polymerase)	12.5	1X
Forward primer(10µM)	1	0.4 µM
Reverse primer(10µM)	1	0.4 µM
DNA template	1	100-300 ng in25 µl
Final reaction volume	25 µl	-

The reaction mixture can be calculated based on the volume of reaction we are going to perform.Likewise, the PCR reactions can also be run in volumes as small as 25 µl. To do this, increase or decrease the volume of the reagents accordingly.

Primer details

Primer	Sequence	Product	References
389F	5' CGG AAC ACA ACC CGA CTT TA 3	389 bp	Tang *et al*. (2000)
389R	5' GGC CAA GAC CAA AAT ACG AA 3'		
77012F	5' ATC GGT GCA CTA CTC GGA 3'	356 bp	Nunan *et al*. (2000)
77353R	5' TCG TAC TGG CTG TTC ATC 3'		
392F	5' GGG CGA ACC AGA ATC ACT TA 3'	392 bp	Tang *et al*. (2000, 2007)
392R	5' ATC CGG AGG AAT CTG ATG TG 3'		
309F	5' TCC AAC ACT TAG TCA AAA CCA A 3'	309 bp	Tang *et al.* (2007)
309R	5' TGT CTG CTA CGA TGA TTA TCC A3'		
MG831F	5' TTG GGG ATG CAG CAA TAT CT 3'	831 bp	Tang *et al*. (2007)
MG831R	5' GTC CAT CCA CTG ATC GGA CT 3'		

PCR cycling conditions

Initial denaturation	94°C for 5 min.	
Denaturation	94°C for 30 sec.	35 Cycles
Annealing	55°C for 30 sec.	
Extension	72°C for 30 sec.	
Final extension	72°C for 5 min.	

It is desirable to carry out the PCR reactions along with a DNA from a known positive sample as positive control, DNA from a known negative tissue sample as negative control and a 'no template' control to make sure that the reaction has taken place properly.

Agarose gel electrophoresis

About 10 µl of the PCR product was electrophoresed in a 1.5% agarose gel (containing 0.5–1 µg/ ml ethidium bromide). The results can be visualized by observing the specific band in the gel under UV transilluminator or any gel documentation system (based on the primers used and their amplicon size).

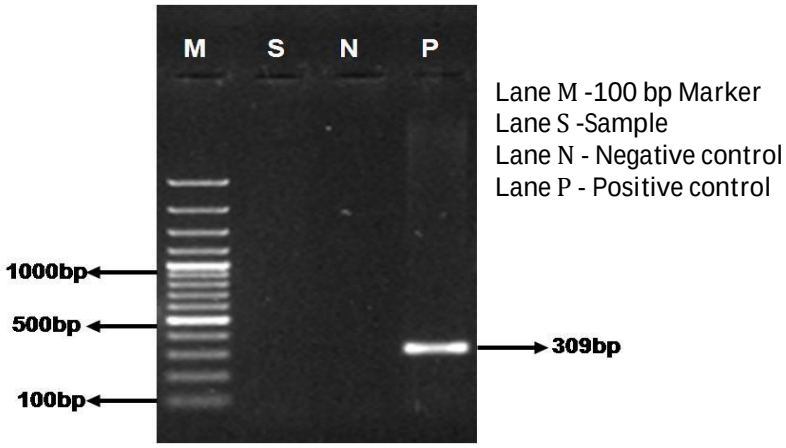

Fig. 1. Agarose gel electrophoresis of the amplified PCR product specific for IHHNV infection

Prevention and Control

- Use of IHHN resistant /(SPF)broodstockfor breeding purpose by hatcheries
- Disinfection of eggs and larvae by hatcheries
- Use of PCR tested seeds for stocking the ponds
- Following a strict biosecurity measures in the farm

References

Duda T.F.Jr. & Palumbi S.R. (1999). Population structure of the black tiger prawn, *Penaeus monodon*, among western Indian Ocean and western Pacific populations. *Mar. Biol.,* 134: 705–710.

Krabsetsve K., Cullen B.R. & Owens L. (2004). Rediscovery of the Australian strain of infectious hypodermal and haematopoietic necrosis virus. *Dis. Aquat. Org.,*61: 153–158.

Nunan L.M., Poulos B.T. & Lightner D.V. (2000). Use of polymerase chain reaction (PCR) for the detection of infectious hypodermal and hematopoietic necrosis virus (IHHNV) in penaeid shrimp. *Mar. Biotechnol.*, 2: 319–328.

Saksmerprome V., Jitrakorn S., Chayaburakul K., Laiphrom S., Boonsua K. & Flegel T.W. (2011). Additional random, single to multiple genome fragments of *Penaeus stylirostris* densovirus in the giant tiger shrimp genome have implications for viral disease diagnosis. *Virus Res.,*160: 180–190.

Tang K.F.J., Durand S.V., White B.L., Redman R.M., Pantoja C.R. & Lightner D.V. (2000). Postlarvae and juveniles of a selected line of *Penaeus stylirostris* are resistant to infectious hypodermal and

hematopoietic necrosis virus infection. *Aquaculture*,190: 203–210.

Tang K.F.J. & Lightner D.V. (2002). Low sequence variation among isolates of infectious hypodermal and hematopoietic necrosis virus (IHHNV) originating from Hawaii and the Americas. *Dis. Aquat. Org.*, 49: 93–97.

Tang K.F.J. & Lightner D.V. (2006). Infectious hypodermal and hematopoietic necrosis virus (IHHNV) in the genome of the black tiger prawn *Penaeus monodon* from Africa and Australia. *Virus Res.*, 118: 185–191.

Tang K.F.J., Navarro S.A. & Lightner D.V. (2007). A PCR assay for discriminating between infectious hypodermal and hematopoietic necrosis virus (IHHNV) and the virus-related sequences in the genome of *Penaeus monodon*. *Dis. Aquat. Org.*, 74: 165–170.

World Organisation for Animal Health (OIE) (2016). Manual of Diagnostic Tests for Aquatic Animals, Fourth Edition. OIE, Paris, France, 1-21pp.

Detection of Infectious Myonecrosis Virus (IMNV) in shrimp by OIE RT-PCR protocol

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Signs and symptoms, severity, economic loss

Infectious myonecrosis is an emerging *P. vannamei* disease, first detected in Brazil during 2004, and later from Indonesia in 2006. IMN disease characterized by acute onset of gross signs including focal to extensive whitish necrotic areas in the striated muscle, especially of the distal abdominal segments and the tail fan, which may become necrotic and reddened similar to the colour of cooked shrimp. Mortalities canrange from 40 to 70% and continue for several days in *P. Vannamei*grow-out ponds and food conversion ratios (FCR) of infected populations increase from normal values of ~ 1.5 to 4.0 or higher. The economic loss in Brazil due to this disease was estimated to be more than US$ 100 million between 2002 and 2006.Outbreaks of IMN with sudden high mortalities may follow stressful events such as capture by cast-net, feeding, sudden changes in salinity or temperature, etc., in early juvenile, juvenile, or adult *L. vannamei* in regions where IMNV is enzootic.

Causative agent

IMN is caused by a putative totivirus which are icosahedral virus of about 40 nm in diameter.

Susceptible host species

The principal host species is *P. vannamei*in which IMNV is known to cause significant disease mortalities. Juveniles and sub-adults of *P. vannamei*, farmed in marine or low salinity brackish water, appear to be more susceptible to IMNV.

Geographical distribution

Reported from north-eastern Brazil and South-East Asia, including Java Island

Target organs

The principal target tissues for IMNV include the striated muscles (skeletal and less often cardiac), connective tissues, haemocytes, and the lymphoid organ parenchymal cells

Disease transmission

IMNV has been demonstrated to be transmitted through cannibalism, via water and vertical transmission from broodstock to progeny is also likely to occur. IMNV may also be transmitted among farms by faeces of seabirds or shrimp carcasses.

Prevention and Control

IMNV is believed to be transmitted vertically (transovarian transmission), even though there are no published reports documenting this route of transmission. Disinfection of eggs and larvae is a good management practice recommended to reduce the potential of IMNV contamination of spawned eggs and larvae produced from them. General husbandry practices have been successfully applied to the prevention of IMNV infections and IMN disease. IMNV may be detected using a nested reverse-transcriptase polymerase chain reaction (RT-PCR) method. The disease can be prevented by stocking with virus free PL produced from IMNV-free broodstock.

Reverse-Transcription Polymerase Chain Reaction (RT-PCR) protocol for detection of IMNV

Sampling for disease diagnosis

Suitable specimens for testing for infection by IMNV include postlarvae (PL), juveniles and adults. While IMNV may infect all life stages, but virus load, may be below detection limits in spawned eggs and in the larval stages, hence these life stages may not be suitable samples for IMNV detection.

Best organs or tissues

IMNV infects tissues of mesodermal origin. The principal target tissues in the acute phase of IMNV infection are the striated muscles (skeletal and less commonly cardiac muscle), connective tissues, haemocytes, and the lymphoid organ tubule parenchymal cells. In chronic infections, the lymphoid organ may be the principal target tissue. Haemolymph or excised pleopods may be collected

and used when non-lethal testing of valuable broodstock is necessary

Pooling of samples

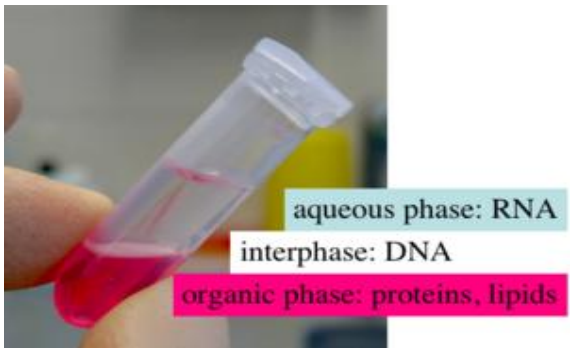
Suitable specimens for testing for infection by IMNV include postlarvae (PL), juveniles and adults. Samples taken for molecular tests may be combined as pooled samples representing no more than five specimens per pooled sample of juveniles, subadults and adults. However, for eggs, larvae and PL, pooling of larger numbers (e.g. ~150 or more eggs or larvae or 50–150 PL depending on their size/age) may be necessary to obtain sufficient sample material (extracted nucleic acid) to run a diagnostic assay.

RT-PCRDiagnosis of IMNV

Isolation of RNA using TRIZOL method

Principle

RNA (Ribonucleic acid) is a polymeric substance present in living cells and many viruses. RNA is used in all the steps of protein synthesis in all living cells and carries the genetic information for many viruses. The isolation of RNA with high quality is a crucial step required to perform various molecular biology experiment. TRIzol Reagent is a ready-to-use reagent used for RNA isolation from cells and tissues. TRIzol works by maintaining RNA integrity during tissue homogenization, while at the same time disrupting and breaking down cells and cell components. Addition of chloroform, after the centrifugation, separates the solution into aqueous and organic phases.RNA remains only in the aqueous phase. After transferring the aqueous phase, RNA can be recovered by precipitation with isopropyl alcohol. Total RNA extracted by TRIzol Reagent is free from the contamination of protein and DNA. This RNA can be used in Northern blot analysis, in vitro translation, poly (A) selection, RNase protection assay, and molecular cloning.



Reagents

- TRIZOL® Reagent
- Chloroform
- Isopropyl alcohol
- 75% Ethanol (in DEPC-treated water)
- RNase-free water or 0.5% SDS solution
- DEPC inactivates the RNases by the covalent modifications of the histidine residues. To prepare RNase-free water, draw water into RNase-free glass bottles. Add diethylpyrocarbonate (DEPC) to 0.01% (v/v). Let stand overnight and autoclave.

RNA Extraction using TRIZOL method

1. RNA templates

1. Frozen or ethanol-fixed tissue (pleopods, cephalothorax, muscle)
2. Haemolymph (weaker reactions than with other tissues)

Tissues Homogenization

Safety Warning–*The chemicals used for RNA isolation are hazardous, avoid skin contact and breathing the vapours. Personal protective clothing, including gloves should be worn at all times when handling TRIZOL reagent*

- *Frozen tissues can be thawed directly in TRIZOL reagent*
- *Ethanol preserved tissue should be blotted dry with a clean tissue and then processed as for fresh tissue*

- *Haemolymph collected into2 volumes of10%sodiumcitratesolutioncan be used directly*
- *The 10mm larvae stored in 95% alcohol weigh approximately 5mg solid Tissue*

Homogenize per 50-100 mg of tissue samples in 1 ml of TRIZOL® Reagent tissue using a homogenizer. Following homogenization, remove insoluble material from the homogenate by centrifugation at 12,000 × *g* for 10 minutes at 4°C. Transfer the clear homogenate solution to a fresh tube and proceed with chloroform addition and phase separation.

2. Phase Separation

- Incubate the homogenized samples for 5 minutes at room temperature (15 to 30°C) to permit the complete dissociation of nucleoprotein complexes.
- Add 0.2 ml of chloroform per 1 ml of TRIZOL® Reagent. Shake tubes vigorously by hand for 15 seconds and incubate them at for 2 to 3 minutes.
- Centrifuge the samples at 12,000 × *g* for 15 minutes at 4°C. Following centrifugation, the mixture separates into a lower red, phenol-chloroform phase, an interphase, and a colorless upper aqueous phase. RNA remains exclusively in the aqueous phase.

3. RNA Precipitation

Transfer the aqueous phase to a fresh tube. Precipitate the RNA from the aqueous phase by mixing with 0.5 ml of isopropyl alcohol. Incubate samples at 15 to 30°C for 10 minutes. Centrifuge at 12,000 × *g* for 10 minutes at 4°C. The RNA precipitate, often invisible before centrifugation, forms pellet on the side and bottom of the tube.

4. RNA Wash

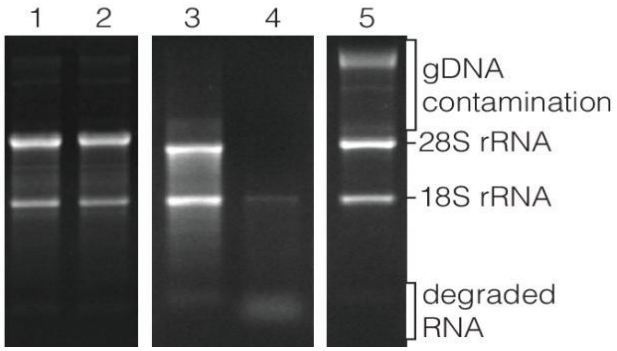
Remove the supernatant. Wash the RNA pellet once with 1 ml of 75% ethanol. Mix the sample by vortexing and centrifuge at 7,500 × *g* for 5 minutes at 4°C.

5. Re-dissolving the RNA

Air dry the RNA pellet. It is important not to let the RNA pellet dry completely as this will greatly decrease its solubility. Dissolve RNA in RNase-free water and incubating for 10 minutes at 55 to 60°C. RNA can also be redissolved in 100% formamide (deionized) and stored at -70°C. The RNA precipitate can be stored in 75% ethanol at 2 to 8°C for at least one week, or at least one year at –5 to -20°C.

Determining RNA quality

The ratio of the readings at 260 nm and 280 nm (A_{260}/A_{280}) provides an estimate of the purity of RNA with respect to contaminants that absorb in the UV, such as protein. However, the A_{260}/A_{280} ratio is influenced considerably by pH. For accurate ratios, it is recommend measuring absorbance in a low-salt buffer with slightly alkaline pH (e.g., 10 mMTris·Cl, pH 7.5). Pure RNA has an A_{260}/A_{280} ratio of 1.9–2.1.



The 28S and 18S rRNA bands are indicated. Lanes 1 and 2 are examples of intact RNA with a 28S:18S rRNA ratio of approximately 2:1. Lane 3 is an example of degraded RNA with RNA smearing below the 28S and 18S rRNA bands. Lane 4 is an example of RNA degradation resulting in the loss of the 28S rRNA band and an accumulation of degraded RNA near the bottom of the gel. Lane 5 is an example of RNA with significant genomic DNA (gDNA) contamination

Quantification of RNA

The concentration of RNA should be determined by measuring the absorbance at 260 nm (A_{260}) in a spectrophotometer. To ensure significance, readings should be between 0.15 and 1.0. An absorbance of 1 unit at260 nm corresponds to 44 µg of RNA per ml (A_{260} = 1 → 44 µg/ml; based on a standard 1 cm path length).

Example of RNA quantitation
Volume of RNA sample = 100 µl
Dilution = 10 µl RNA sample + 490 µl of 10 mMTris·Cl, pH 7.0 (1/50 dilution)
Measure absorbance of diluted sample in a 1 ml cuvette (RNase-free)
A_{260} = 0.2

RNA concentration
= 54 µg/ml x A_{260} x dilution factor
= 54 µg/ml x 0.2 x 50
= 540 µg/ml

Total amount of RNA
= concentration x volume of sample in ml
= 540 µg/ml x 0.1 ml
= 54 µg RNA

Precautions for RNA extraction

RNAses can be introduced into the RNA preparation during the isolation procedure. To reduce this, Always wear disposable gloves. Use sterile, disposable plastic ware and automatic pipettes reserved for RNA work to prevent cross-contamination.

Storage

RNA may be stored in, RNase-free waterwith 0.1 mM EDTAor TE buffer (10 mM Tris, 1mM EDTA) for short term use. RNA is generally stable at -80° C for up to a year without degradation.. It has been suggested that RNA solubilized in formamide may be stored at -20°C for at least one year (Chomczynski, 1992). For long term storage, RNA samples may also be stored at -20°C as ethanol precipitates.

Nested PCR for detection of Infectious Myonecrosis virus (IMNV) –OIE Protocols

Introduction

A nested RT-PCR method was developed for the detection of IMNV using two primer sets that produce 328 and 139 bp amplicons. This method for detection of nucleicacid (RNA) from IMNV is based on the method outlined in the OIE Manual of Diagnostic Tests for Aquatic Animals 2016 -Chapter 2.2.4 . – Infectious myonecrosishttp://www.oie.int/index.php?id=2439&L=0&htmfile=chapitre_infectious_myonecrosis.htmand is based on the publications ofLightner (2005) and PoulosandLightner(2006).

Procedure from OIE Protocol

The method is summarised below: Template must be boiled for 3 minutes and put on ice just prior to adding to reaction mix

OIE outer Primers (First step)

Primer	Sequence (5' to 3')	Amplicon Size	Ref.
4587F	GACGCTGCTAACCATACAA	328 bp	Lightner (2005)
4914R	CTCGGCTGTTCGATCAAGT		

OIE inner Primers (second step)

Primer	Sequence (5' to 3')	Amplicon Size	Ref.
4725 NF	GGCACATGCTCAGAGACA	139bp	Lightner (2005)
4863 NR	AGCGCTGAGTCCAGTCTTG		

OIE Reaction Mixture –First Step (25 µl reaction volume)

Reagent	25 µl reaction	Final concentration
DD H_2O	6.5 µl	-
5 × EZ Buffer	5.0 µl	1 ×
dNTP mix (10mM each)	3.0 µl	300 µM each
Primer F (100 ng µl–1)	1.0 µl	0.62 µM
Primer R(100 ng µl–1)	1.0 µl	0.62 µM
Mn(Oac)2 (25 mM)	2.5 µl	2.5 mM
rTth Enzyme (2.5 U µl–1)	1.0 µl	0.1 U µl–1
Template	1–5 µl	1–50 ng total RNA

OIE Reaction Mixture – Second Step (25 µl reaction volume)

Nested PCR reaction (Amersham Biosciences pure Taq Ready To Go Beads #27-9558-01):

Template for the nested reaction is the product from the first step reaction

Reagent	25 µl reaction	Final concentration
DD H2O	22.5 µl	-
Primer NF-(100 ng μl^{-1})	1.0 µl	0.465 µM
Primer NR (100 ng μl^{-1}	1.0 µl	0.465 µM
Template	0.5 µl	-

OIE Cycle Conditions - First step

Primers	Temperature (°C)	Time	No. cycles	Amplicon
4587F/ 4914R	60, 95	30 minutes, 2 minutes	1	328 bp
	95, 60	45 seconds, 45 seconds	39	
	60	7 minutes	1	

OIE Cycle Conditions - Second step

Primers	Temperature (°C)	Time	No. cycles	Amplicon
4725 NF/ 4863 NR	95	2 minutes	1	139 bp
	95, 65, 72	30 seconds, 30 seconds, 30 seconds	39	
	72	72minutes	1	

Analysis

10 µl of the amplified PCR product is added to a well of a 1.0% agarose gel in 0.5 x TBE (Tris, boric acid, ethylenediamine tetra-acetic acid EDTA])containing ethidiumbromide (10ml of 10mg/ml EtBr stock in200ml agarose solution), (final conc = 0.5mg/ml).

5 µl of 100bp DNA ladder is used as a marker.

Gel pictures of First and second step IMNV

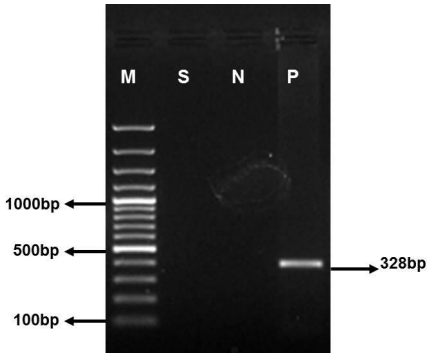


Fig.1Agarose gel picture showing detection of IMNV by first stepRT-PCR
Lane M- 100bp Marker;Lane S-Sample (Negative); N- Negative control; P-Positive Control.
Expectedamplicon size: 328 bp

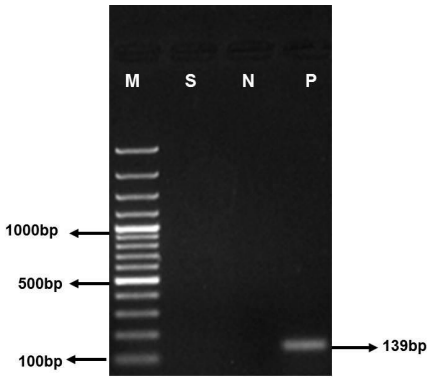


Fig.2 Agarose gel picture showing detection of IMNV by nested RT-PCR
Lane M- 100bp Marker; Lane S-Sample (Negative); N- Negative control; P-Positive Control.
Expectedamplicon size: 139 bp

Interpretation of Result

First Step

The IMNV-specific amplicon from this reaction is 328bp.The analytical sensitivity is approximately 100copies of a IMNV plasmid template.

Second Step

The IMNV -specific amplicon from this reaction is 139bp.The overall sensitivity of both steps is approximately 10 copies of a IMNV plasmid template

Trouble shooting

The input amount of RNA is not optimal for RT reaction

Several factors including sample quality during collection, processing, and storage influence the isolation and concentrations of RNA from sample

- Homogenize samples immediately after harvesting in cell lysis solution (e.g., containing guanidinium), Flash freeze samples in liquid nitrogen or collection of samples in RNA*later*® orRNA stabilization solution are effective methods to prevent RNA degradation.
- Flash-frozen samples must be stored at –80°C and tissues should be homogenized in lysis buffer directly from the frozen state, or ground or pulverized at cryogenic temperatures prior to homogenization in a lysis solution.
- Thorough method of homogenization tailored made to the cell or tissue type essential to prevent both RNA loss and RNA degradation.
- Additional treatments are needed for some samples after homogenizationfore.g., tissues like brain and adipose tissue, high in fat, should be extracted with chloroform to remove lipids and increase RNA yields
- For short-term storage, resuspended RNA should be stored at –20°C; for long-term storage, it should be stored at –80°C.
- Although RNA resuspended in water or buffer can be stored at –80°C, RNA is most stable in an NH_4OAc/ethanol precipitation mixture at –80°C , in multiple aliquots to prevent RNA damage from successive freeze-thawing and accidental RNase contamination
- To isolate intact, high-quality RNA, it is essential that RNases are not introduced into RNA preparations. RNases are found almost everywhere, and it is essential that purified RNA to be RNase-free. All surfaces, including pipettes, benchtops, glassware, and gel equipment, should be decontaminated with a surface decontamination solution. RNase-free tips, tubes, gloves and solutions should always be used.

Inhibitors present in RNA

It is of fundamental importance not only to stabilize the RNA, in order to prevent RNA degradation by RNases, but also to select the extraction method that can assure a high yield and purity of nucleic acid and a complete removal of PCR inhibitors. The extraction method select should take into account not only the starting material, but also the relative RNase activity and nucleic acid content

RNA Integrity

The integrity of the RNA is important for the yield. RNA quality will have impact on the results of cDNA synthesis and batch to batch variation in RNA quality will lead to inconsistent results. Before performing an RT, check the quality of RNA by running an agarose gel. Intact RNA should demonstrate 28s and 18s rRNA with the larger band double in intensity compared to the smaller.

Secondary Structure in RNA

RNA secondary structure can be a problem in transcribing full length RNA. The RT enzyme can stop when it hits loop structures in the RNA. It is difficult to know template RNA will have secondary structure, but the gene of interest with high GC content might indicate difficult to melt apart RNA completely. For this reason, a usual first step is a 5 minute 65C denaturation to melt apart the RNA. Alternatively, there are RT enzymes with higher efficiency at moving through secondary structure, even at standard RT incubation temperatures (37^0C-42^0C).

Quantification of RNA

The accuracy of the yield can be affected by contamination with DNA, contamination with salts,

and level of degradation. For measuring the yield, use UV quantification using the Nanodrop

False positives

Genomic DNA contamination in the RNA can cause false positives in the final PCR. Hence DNase treatmentis recommend to remove residual genomic DNA contaminationeither duringafterRNA Preparation.

References

1. Lightner D.V. (2005). Biosecurity in shrimp farming: pathogen exclusion through use of SPF stock and routine surveillance. J. World Aquaculture Soc., 36, 229–248

2. Poulos B.T. andLightner D.V. (2006). Detection of infectious myonecrosis virus (IMNV) of penaeid shrimp by reverse-transcriptase polymerase chain reaction (RT-PCR). Dis. Aquat. Org., 73, 69–72.

Detection of Infectious Myonecrosis Virus (IMNV) by RT-PCR using commercial Kit

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1.0 Introduction

IMNV is a *Totivirus*. Phylogenetic analysis of its RNA-dependent RNA polymerase (RdRp) gene coding sequence indicates that IMNV is most closely related to *Giardia lamblia* virus, a member of the family *Totiviridae*. IMNV particles are icosahedral in shape and 40 nm in diameter, with a buoyant density of 1.366 g ml–1 in caesium chloride. The genome consists of a single, double-stranded (ds) RNA molecule of 7560 bp. IMN disease is not the same disease as white tail disease of Penaeid shrimp and white tail disease of *Macrobrachium rosenbergii*. The principal host species in which IMNV is known to cause significant disease outbreaks and mortalities in farmed populations is *Penaeus vannamei*

The Pacific blue shrimp, *P. stylirostris*, and the black tiger shrimp, *P. monodon* have been infected experimentally with IMNV. Juveniles and sub adults of *P. vannamei*, farmed in marine, brackish, and low salinity brackish water, appear to be most severely affected by IMN disease. IMNV has been demonstrated to be transmitted horizontally by cannibalism. Transmission via water and vertical transmission from broodstock to progeny probably occurs. Although vertical transmission is suspected from anecdotal evidence, it is not known whether this occurs via transovarial mechanism or by surface contamination of newly spawned eggs.

2.0 Sample Preparation

2.1 Requirements

- Micro Centrifuge tubes
- RNA Later or 95 % Ethanol
- Forceps, Scalpel, Scissors&Blade
- Sodium Hypo chloride (5%)

2.2. Processing Shrimp for Nucleic Acid Extraction

This section provides sample dissection procedures for different life cycle of Shrimp. After sampling is carried out follow the Nucleic acid extraction protocol as given in following sections. If samples preserved in Ethanol, dry completely before nucleic acid extraction.

2.2.1 Brood stock eye stalk

- Use the whole eye stalk and put into a 1.5 ml micro centrifuge tube.

2.2.2 Larvae, PL or Juvenile

- Place about 50 mg specimen into a 1.5 ml micro centrifuge tube. The test requires at least 50 pieces for larvae; 30 pieces for <PL12; for >PL12 use 30 tail pieces, only half tail should be used.

2.2.3 Pleopod & gill of Adult shrimp

- Place a piece of Pleopod/gill (50 mg) into a 1.5 ml micro centrifuge tube.

2.2.4 Tail muscle of Adult shrimp

- Place tail muscle (50 mg) into a 1.5 ml micro centrifuge tube.

2.2.5 Shrimp Egg &Nauplii

- Place 20 – 30 mg Egg or Nauplii sample into a 1.5 ml micro centrifuge tube.

3.0 RNA Extraction from Shrimp

3.1 Equipment required

- Cooling Centrifuge
- Vortex Mixer
- Laminar air flow chamber

3.2 Materials & Reagents required

- 1.5 ml Micro centrifuge tubes
- IQ 2000 RNA Extraction Kit
- Isopropanol
- 75 % Ethanol

- Chloroform
- Diethyl pyro carbonate (DEPC) treated Water
- Micropipette and Micro tips (ART)

3.3 IQ 2000 RNA Extraction

- Put sample into a 1.5 ml tube with 500 µl RNA Extraction Solution and the sample with grinding sticks
- Incubate in room temperature for 5 minutes.
- Add 100 µl of $CHCl_3$ then vortex 20 seconds. Incubate in room temperature for 3 minutes, and then centrifuge it at 12000 rpm for 15 minutes.
- Transfer 200 µl of the upper clear aqueous phase to a fresh 1.5 ml tube with 200 µl 2-propanol (Isopropanol)
- Vortex briefly, centrifuge at 12000g for 10 minutes, then discard the Isopropanol.
- Wash the pellet with 500 µl 75% ethanol, then spin down 5 minutes by 9000 rpm to recover RNA pellet, then discard the ethanol and dry the pellet.
- Dissolve the pellet with DEPC water

4.0 Dissolution of RNA

4.1 Material required

- DEPC Water
- Micro centrifuge tubes
- Pipettes and Micro tips (ART)

4.2 Dissolution of RNA

A. The yield of RNA is different for different samples; therefore the concentration of RNAshould be adjusted by dissolving the RNA pellet in different volume of DEPC Water

Sample Source	Volume
Eye stalk of brood stock	100 µl
Post Larvae (PL)	200 µl
Pleopod or Periopod	200 µl
Gill	200 µl
Tissue / Muscle	300

B. Adjust the volume of DEPC Water according to the quality and quantity of the individual samples measured by using the spectrophotometer.

C. These RNA samples could be stored at – 20 ^{o}C.

4.3 Quality and Quantity measurement of Extracted RNA

Equipment required

- Bio photometer
- Vortex Mixer

4.4 Materials required

- Cuvette
- Pipettes and Micro tips (ART)
- DEPC Water

Quality and quantity of RNA is measured using spectrophotometer. Approximately 50 µg/ml with the absorption ratio of 260/280 nm and the range is 1.7 to 2.0.

5.0 Amplification of RNA

5.1 Equipment required

- Thermo cycler
- PCR Workstation
- Vortex mixer
- Spin down centrifuge

5.2 Materials & Reagents requirement

- IQ2000 PCR Kit for IMNV
- 0.2 ml PCR tubes
- PCR Cooler racks for 1.5 ml micro centrifuge tubes and 96-well 0.2ml PCR reaction plates
- Sterile nuclease-free 1.5 ml& 0.2 ml micro centrifuge tubes
- Positive Controls & Negative Controls

Amplification Procedure - A

Standard PCR Protocol

A. First PCR Reaction reagent mixture: 8 µl reaction

First PCR Premix	7.0 µl
RT Enzyme Mix	0.5 µl
IQZymeRNA Polymerase (2U/µl)	0.5 µl

B. Nested PCR reaction reagent mixture: 15µl/reaction

Nested PCR Premix	14 µl
IQzyme RNA Polymerase (2U/µl)	1 µl

Reaction Procedure

- Prepare first PCR reaction reagents as per the no. of samples and standards to be tested.
- Pipette 8 µl of PCR reaction reagent mixture into each tube with proper label.
- Add 2 µl of the extracted sample DNA and standard into each tube with reaction mixture. Perform first PCR reaction.
- Prepare nested PCR reagent mix as per the no. of samples to be tested.
- Add 15 µl of nested PCR reaction reagent mixture to each tube after first PCR was completed. Perform nested PCR reaction.

Reaction condition

Premix:

Reaction step	Temp	Time	Cycles
Reverse Transcription	42°C	30 min	1
Initial Denaturation	94°C	2 min	1
Denaturation	94°C	20 sec	15
Annealing	62°C	20 sec	
Extension	72°C	30 sec	
Final Extension	72°C	30 sec	1

Nested Mix

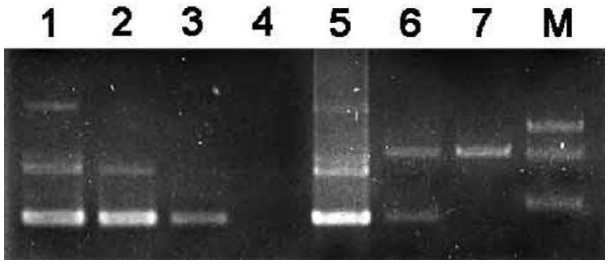
Reaction step	Temp	Time	Cycles
Denaturation	94°C	20 sec	30
Annealing	62°C	20 sec	
Extension	72°C	30 sec	
Final Extension	72°C	30 sec	1
Hold	20°C	30 sec	1

6.0 Electrophoresis

- Prepare 10 X TBE or TAE and dilute to 1X concentration.
- Prepare 2% agarose gel in a conical flask.
- Cool down the clear Agarose gel under room temperature about 25°C.
- Add 2 µl of 10 mg/ml Ethidium Bromide for 100 ml of agarose gel and mix completely then slowly pour the gel into the casting tray. Allow the gel to set/solidify.
- The height of Agarose gel should only above the bottom of the gel comb for about 0.3~0.5 cm.
- Load 10~15µl of the PCR product with loading dye into each well.
- Add 5µl DNA markers for every batch. The DNA molecular weight marker provides a reference for the DNA size and position.
- After sample loading, connect the gel box with power supply before switching on. Adjust the voltage to 60~100 V.
- The 6X loading dye contains 2 colorants: Bromophenol Blue (deep blue colour) and Xylene Cyanole FF (light blue colour). Switch off the power supply when the deep blue colorant approaches 1/2 to 2/3 of the gel.
- Record the results and label the image using Gel Documentation System.
- Place the gels into 5% bleach solution overnight before discard.

Diagnosis

Positive samples and standards will show the following patterns on gel:



Lane 1: IMNV P(+) standard, 2000 copies/reaction
Lane 2: IMNV P(+) standard, 200 copies/reaction
Lane 3: IMNV P(+) standard, 20 copies/reaction
Lane 4: Negative control (Yeast tRNA or ddH_2O)
Lane 5: Sample of severe IMNV infection
Lane 6: Sample of light IMNV infection
Lane 7: IMNV negative sample
Lane M:Molecular weight marker, 848 bp, 630 bp, 333 bp

2. Negative samples will show only one band at 680 bp, which is a PCR product of housekeeping gene as a internal control.
3. Diagnostic procedure:
    a. Band formed at 255 bp and/or 510 bp: Postive
    b. Band formed only at 680 bp: Negative

Detection of AHPND in shrimp using PCR

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Introduction

Acute hepatopancreatic necrosis disease (AHPND) is the most important bacterial disease threat for cultured shrimp. It is usually characterized by mortality ranging from 40–100 % during the first 35 days of culture. The disease was initially named as early mortality syndrome (EMS). There were reports that China lost 80 % of their production due to this disease. Very high economic loss was also reported in Vietnam and Thailand with severe mortality till 2014. Since AHPND is an emerging pathogen, knowledge on pathogenesis is not completely understood. However, the recent research from different parts of the world is unravelling the mystery of this disease and the gene responsible for toxin production. At present, India is free from this disease. But, emergence of new strain of AHPND in South America and elsewhere demands to formulate effective disease control strategy to prevent its occurrence in Indian farm. Confirmation of AHPND outbreak includes isolation of AHPND bacteria, genomic and molecular detection by PCR and histopathological changes at the acute stage of infection.It is very important to differentiate the early mortality caused by AHPND and Non-AHPND causes ranging from environmental factors to various other pathogens.

Signs and symptoms

The AHPND affected shrimp shows lethargy, reduced feed intake and growth rate, spiral swimming, empty stomach and empty gut. The affected shrimp finally sinks and die at the pond bottom. Clinical symptoms include atrophy and pale discolouration of hepatopancreas, at times black discolouration as spots or streaks of hepatopancreas were reported.

Causative agent

Aetiological agent was identified in the year 2013, as of bacterial origin with immersion studies and Koch's postulates were satisfied by isolating a specific virulent strain of *Vibrio parahaemolyticus* (VP_{AHPND}). The VP_{AHPND} strain is a Gram negative bacterium similar to *Vibrio parahaemolyticus* species found in estuarine and marine environment. But the particular strain contains one or more extrachromosomal plasmids involved in expression of plasmid encoded toxin capable of inducing AHPND histopathology and mortality in shrimp. The VP_{AHPND} plasmid was designated as pVPA3-1 and its plasmid encoded toxin as Pir^{vp}. The toxin is homologous to the Pir, Photorhabdus insect related binary toxin. This Pir^{vp}toxin is made of two subunits, $PirA^{vp}$ and $PirB^{vp}$. The subunit $PirB^{vp}$ alone is capable of producing pathological changes of AHPND, whereas $PirA^{vp}$ causes only minor histological changes.

Susceptible host species

The AHPND bacterium was reported to cause infection in Pacific white shrimp (*Penaeus vannamei*), Black tiger shrimp (*Penaeus monodon*) and *Penaeus chinensis*.

Geographical distribution

This disease was first noticed in cultured shrimp farms of China during 2009 and it spread to other countries like Vietnam (2010), Malaysia (2011), Thailand (2012) and Mexico (2013). The disease was initially named as early mortality syndrome (EMS) due to the mass mortality and loss of culture during the initial days after stocking. A pathogenic *Vibrio parahaemolyticus* strain was isolated from AHPND affected shrimp from South America. Its genomic analysis revealed the strain is more related to strains of South East Asia as compared to

Mexico. In India the disease has not yet been reported.

Target organs

The target tissue of AHPND bacteria is hepatopancreas starting from proximal to distal part of the organ. It disrupt mitotic cell division of E-cells, dysfunction of R, B, F cells with massive cell rounding and sloughing of hepatopancreatic tubule epithelial cells.

Disease transmission

Transport of PL from infected to non-infected region play an important role in the spread of AHPND pathogens. During experimental infections, mortality can be induced within 12 hours of exposure to strains of $VP_{AHPND}$by the *per os* route if the coated feed contains 10^8 CFU (colony-forming units) per gram of inoculum (Nunan *et al.,* 2014; Soto-Rodriguez *et al.,* 2015; Tran *et al.,* 2013b).Isolation of live *V. parahemolyticus* and detection by molecular methods were unsuccessful on frozen shrimp samples maintained at -18^{o}C to -24^{o}C for several weeks. Similarly, refrigeration of samples at 4 ^{o}C can lead to reduction of culturable bacterial after storage for several weeks. The *V. parahemolyticus* is sensitive to heat and get killed by heating at 55 ^{o}C for five minutes and 80 ^{o}C for 1 minute. It is susceptible to common disinfectants and at gets killed after treatment at pH 5 for 5 minutes.

Prevention and Control

Brookstock should be kept in quarantine and tested for AHPND before spawning. Avoid excel feeding; provide optimal quantity of feed. Test the larvae for AHPND / EMS by PCR before stocking. Monitor theponds regularly, particularly during the early periods. Follow strict biosecurity measures; avoid use of common water body, use reservoir ponds, bird fencing etc. Adopt closed re-circulatory system or zero water exchange practice to avoid contamination. Include nursery phase to grow shrimps postlarvae in nursery before stocking larger size juveniles in culture ponds. Using biofloc technology in shrimp culture appears to be useful in preventing AHPND/EMSoutbreak. Co-culture of tilapia and shrimp or culture with tilapia induced green water would help to reduce AHPND incidence.

PCR Diagnosis

Sample collection

The moribund shrimps showing clinical signs should be collected and sent to the laboratory in live condition will be ideal for isolation of bacteria, molecular detection and histopathological studies. Post larval specimens can be pooled but larger shrimp tissues should be processed individually. Gut associated tissues and organs like hepatopancreas, stomach and gut should be collected. In case of broodstock faecal material can be collected for diagnosis. For molecular identification the above samples should be collected in 90% ethanol and for histopathology the tissues should be preserved in Davidson's fixative.

DNA preparation for PCR analysis

Shrimp gut associated tissues or bacterial pellets from fresh tryptic soya broth (1.5% NaCl) should be homogenized in lysis buffer (50mM Tris-HCl pH 8.0, 50 mM EDTA, 1% SDS, 10mM NaCl) containing 5mg ml^{-1} proteinase K. The genomic DNA can be isolated and further purified by phenol-chloroform method (Sambrook and Russel, 2001). Finally, the extracted DNA should be checked for concentration using Nanodrop and optimally adjusted to 50 ng/µL concentration with TE buffer (pH 8.0) for PCR analysis. The extracted DNA can be kept for long period at 4 ^{o}C.

PCR methods to detect AHPND

The PCR detection of AHPND is the fastest and very economical method of disease diagnosis. Various PCR based kits are commercially available in the market since 2013. Initially, AP1 and AP2 primers were developed based on DNA plasmid sequences. Subsequently AP3 primer version was developed based on sequences of VP_{AHPND} TOX A gene (NACA, 2014). All the above three primers were based on 1-step PCR detection technique which lacked the sensitivity to detect the low level

infection of AHPND. Therefore, it required initial enrichment of bacteria in suitable media which is not possible with frozen or alcohol preserved samples.Recently Dangtip et al. (2015) has developed a nested PCR protocol using AP4 primer. The AP4 primer based nested PCR method is 100 times more sensitive than previous version. The target sequence for primer AP4 is located on ToxA and ToxB region on the pVA plasmid (69 kbp) of VP_{AHPND} isolates. The first step PCR target 1269 bp ToxA and Tox B gene sequences. Second step nested primers AP4-F2 and AP4-R2 target 230 bp sequence (Table 1).

After preparing the reaction mix, keep the tube to a thermocycler. Set the PCR reaction condition as per cycling condition mentioned in Table 2.

Table 1. The AP4 primer sequence

Primers	5'-3'	PCR Product size	Reference
AP4-F1	ATG AGT AAC AAT ATA AAA CAT GAA AC	1269 bp	Dangtip et al. 2015
AP4-R1	ACG ATT TCG ACG TTC CCC AA		
AP4-F2	TTG AGA ATA CGG GAC GTG GG	230 bp	
AP4-R2	GTT AGT CAT GTG AGC ACC TTC		

Table 2. PCR reaction condition for AP4 primer

PCR step	Ist step PCR	IInd step PCR (nested PCR)
Initial denaturation	94 ^{o}C, 2 min	94 ^{o}C, 2 min
Denaturation	94 ^{o}C, 30 sec	94 ^{o}C, 20 sec
Annealing	55 ^{o}C, 30 sec	55 ^{o}C, 20 sec
Extension	72 ^{o}C, 90 sec	72 ^{o}C, 20 sec
Final extension	94 ^{o}C, 2 min	72 ^{o}C, 2 min
No. of cycle	30	25

The amplified PCR product is detected in 1% agarose gel prepared in 1X TAE buffer with ethidium bromide (0.5 µg/ml). The amplified products (5 to 10µl) are mixed with 6× gel loading buffer and added to individual wells of the gel. Run a 1 kb or 100 bp marker as a standard to know the amplified product size. Run the gel in 1X TAE buffer for about 1 hour at 80-100 V alongwith keeping observation on dye movement. Observe the gel in gel documentation system, see for PCR product of desirable size and record the result.

Conclusion

Till date APHND has not been reported in India. But seeing its severity of infection utmost care should be taken to prevent its entry into the Indian farm. This can be achieved by active collaboration between shrimp farmers, researchers and brooder importing agency. The farmers are suggested to inform the premier agency like CIBA, if high level of mortality is reported in shrimp farm during early stage of culture.

Reference

1. Tran L, Nunan L, Redman RM, Mohney LL, Pantoja CR, Fitzsimmons K, et al. 2013. Determination of the infectious nature of the agent of acute hepatopancreatic necrosis syndrome affecting penaeid shrimp. Dis Aquat Org. 105:45–55.
2. Nunan L, Lightner D, Pantoja C, Gomez-Jimenez S. 2014. Detection of acute hepatopancreatic necrosis disease (AHPND) in Mexico. Dis Aquat Org. 111:81–86.
3. Soto-Rodriguez SA, Gomez-Gil B, Lozano-Olvera R, Betancourt-Lozano M, Morales-Covarrubias MS. 2015. Field and experimental evidence of Vibrio parahaemolyticus as the causative agent of acute hepatopancreatic necrosis disease of cultured shrimp (Litopenaeus vannamei) in Northwestern Mexico. Appl Environ Microbiol. 81: 1689-1699.

4. Joshi J, Srisala J, Truong VH, Chen IT, Nuangsaeng B, Suthienkul O, et al. 2014. Variation in Vibrio parahaemolyticus isolates from a single Thai shrimp farm experiencing an outbreak of acute hepatopancreatic necrosis disease (AHPND). Aquaculture. 428–429:297–302.
5. Flegel TW, Lo CF. Free release of primers for specific detection of bacterial isolates that cause acute hepatopancreatic necrosis disease (AHPND). Network of Aquculture Centres in Asia-Pacific (NACA).
6. Gomez-Gil B, Soto-Rodríguez S, Lozano R, Betancourt-Lozano M. 2014. Draft genome sequence of Vibrio parahaemolyticus strain M0605, which causes severe mortalities of shrimps in Mexico. Genome Announc. 2(2).
7. Sambrook J, Russell DW. Molecular cloning:a laboratory manual 3rd Edition. Cold Spring Harbor: Cold Spring Harbor Press; 2001
8. Dangtip S, Sirikharin R, Sanguanrut P, Thitamadee S, Sritunyalucksana K, Taengchaiyaphum S, Mavichak R, Proespraiwong P and Flegel T.W. 2015. AP4 method for two tube nested PCR detection of AHPND isolates of Vibrio parahaemolyticus. Aquaculture reports. 158-162.
9. Restrepo L, Bayot B, Betancourt I, and Pinzon A. 2016. Draft genome sequence of pathogenic bacteria Vibrio parahaemolyticus strain Ba94C2, associated with acute hepatopancreatic necrosis disease isolate from South America. Genomic Data. 143-144.

Detection of *Enterocytozoonhepatopenaei*(EHP)infection in shrimp using PCR.

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Introduction

Enterocytozoon hepatopenaei (EHP), the causative agent of Hepatopancreatic microsporidiasis has become a major concern for the shrimp farming industry. It is an emerging microsporidian parasite for penaeid shrimp, which has been associated with growth retardation and significant losses in several shrimp farming countries in Asia. More recently, in India the epizootics of Enterocytozoon hepatopenaei have been reported with several disease outbreaks. EHP has been found in the tubules in the shrimp's hepatopancreas and associated with damages in the organ which eventually may lead to metabolism and growth retardation in shrimp.

Gross signs

EHP infection cannot be determined by visual inspection and there are no specifically distinctive gross signs. It is suspected to be associated with growth retardation.

Susceptible Host species

EHP infects *P. monodon* and *P. vannamei* and is suspected to infect *P. Japonicus*

Geographical distribution

EHP has been found in several shrimp farming countries in Asia including Vietnam, Thailand, Malaysia, Indonesia, China and India

Target organs

Shrimp hepatopancreas is the target organ for the detection of EHP infection. Feacal threads can also be used for non-lethal screening of SPF broodstocks. In any case, please note that pleopods or uropds are not the tissue of choice for the PCR diagnosis of EHP

Transmission

The infection can be transmitted horizontally, through oral route by consumption of infected tissue (cannibalism, Predation etc.) and possibly vertical transmission (trans-ovum).

Prevention and control

The life cycle and underlying transmission mechanism of this microsporidian has been poorly understood. There is no specific treatment available for the control of EHP infection. Better management practices (BMPs) are the only way to prevent the epidemics of EHP. In Hatcheries by using EHP free live feeds and by following complete disinfection of hatchery facility with 2.5 % sodium hydroxide solution (with minimum contact time – 3 hrs) followed by a week drying then rinsing with acidified chlorine(200 ppm).

In grow out system by stocking EHP free seed and by following proper pond preparation, before stocking and after harvest to ensure that the EHP spores along with the carriers were destroyed.

PCR protocol for detection of *Enterocytozoonhepatopenaei* in shrimp

EHP is not an OIE listed disease. PCR protocol using primers designed based on small sub unit ribosomal RNA (SSU rRNA) gene and spore wall protein gene has been reported by various researchers. Recently, many commercial kits are also available. While participant laboratories are free to use whatever test system they used in usual practice, it was recognized that most molecular testing is based on conventional nested PCR. We practice the following protocol in our lab,which is being used widely by researchers and adopted by NACA for routine diagnosis of EHP in shrimp. Here, basic straight-forward PCR protocols based on SSU-rRNA gene and spore wall protein geneare

presented. These methods are suitable for testing broodstock, PLs, and their live feeds for EHP infection. Where appropriate, some of the choices for modifying this standard reaction that are routinely available to diagnostic laboratory are presented.

Sample collection

For PCR detection, fresh or ethanol-preserved samples of PL from hatchery or whole hepatopancreas in case of grow-out monitoring may be used. As the microsporidian spores are expelled out through faeces, fresh faecal sample from precious or specific pathogen-free (SPF) broodstock may also be used as non-lethal sample for analysis.

Step 1: Choosing Target Substrates and PCR Primers

The choice of the target DNA is, of course, dictated by the specific purpose. However, one thing is common to all substrate DNAs and that is they must be as clean as possible and uncontaminated with other DNAs. In case of EHP, which infects primarily the hepatopancreas of shrimp, we recommend the use of shrimp hepatopancreas as the preferred tissue of choice in case of farmed shrimp. Most of the primers are based on the SSU rRNA gene or spore wall protein gene of the microsporidian. Naturally, if the source material is an environmental sample such as water or soil, then the researcher must rely upon the specificity of the PCR primers to avoid amplification of the nonspecific target. In case of precious broodstock, nonlethal samples like faecal strings may be collected.

Table 1. Oligonucleotide primers used for PCR assay at CIBA for the detection of *Enterocytozoonhepatopenaei*

Primers	Sequence (5' to 3')	Ampliconsize (bp)	Reference
First step			
ENF 779	CAGCAGGCGCGAAAATTGTCCA	779	Tangprasittipap*et al*. (2013)
ENR 779	AAGAGATATTGTATTGCGCTTGCTG		
Nested			
ENF 176	CAACGCGGGAAAACTTACCA	176	Tangprasittipap*et al*. (2013)
ENR 176	ACCTGTTATTGCCTTCTCCCTCC		
First step			
SWP 1F	TTGCAGAGTGTTGTTAAGGGTTT	514	Itsathitphaisarn*et al*. (2016)
SWP 1R	CACGATGTGTCTTTGCAATTTTC		
Nested			
SWP 2F	TTGGCGGCACAATTCTCAAACA	148	Itsathitphaisarn*et al*. (2016)
SWP 2R	GCTGTTTGTCTCCAACTGTATTTGA		

Step 2: Setting Up the Reaction

Once we choose the appropriate substrate DNA and PCR primer sequences, the basic reaction components are as follows. The role that each of these components plays in a PCR reaction has been discussed earlier.

Water
10x Reaction Buffer
$MgCl_2$
dNTPs
Forward Primer
Reverse Primer
Target DNA
Polymerase enzyme

Take care not to cross-contaminate the reagents, especially the templates and primers.Pipetting order in general; we may add water first and the enzyme last. For a large number of reactions, it is good practice to first set-up a master mix of the common reagents and then aliquot them, rather than to pipette the reagents separately for each individual tube.An example for a typical reaction of 25µl set up is shown below:

Reagents	Vol(µl)	Vol (µl) (x)	Final Conc.
Water	9.5		-
2x Master Mix (contains Buffer with $MgCl_2$, dNTPs, Taq DNA polymerase)	12.5		x1
Forward primer(10µM)	1		0.4 µM
Reverse primer(10µM)	1		0.4 µM
DNA template	1		100-300 ng in 25µl
* Original Rxn.Vol. 25 µl	25 µl		

Step 3: Choosing the Reaction Conditions

The reaction conditions of PCR amplification are composed of the total number of cycles to be run and the temperature and duration of each step in those cycles. The decision as to how many cycles to run is based upon the amount of DNA target material you start with as well as how many copies of the PCR product (amplicon) you want. In general, 25 to 35 cycles is the standard for a PCR reaction. This results in from approximately 34 million to 34 billion copies of the desired sequence using 25 cycles and 35 cycles respectively. Additional cycle numbers can be used if there is a small amount of target DNA available for the reaction. However, reactions in excess of 45 cycles are quite rare. Also, increasing the number of cycles for larger amounts of starting material is counterproductive because the presence of very high concentrations of the PCR product is itself inhibitory.

Once the number of cycles is selected, it is necessary to choose the temperature and duration of each step in the cycles. The first step is the DNA denaturation step that renders the entire DNA in the reaction single stranded. This is routinely accomplished at 94°C or 95°C for 30 seconds. The second step is the primer annealing step during which the PCR primers find their complementary targets and attach themselves to those sequences. Here the choice of temperature is largely determined by the melting temperature (Tm) of the two PCR primers. Again, the usual duration is 30 seconds. Finally, the last step in a PCR cycle is the polymerase extension step during which the DNA polymerase is producing a complimentary copy of the target DNA strand starting from the PCR primer sequence (thus the term primer). The usual temperature of this step is 72°C, considered to be a good optimum temperature for thermal-stable polymerases. A common rule of thumb for the duration of this step has been 30 secondsfor every 500 bases in the PCR product. However, with the increasing quality of commercially available polymerase enzymes and the associated reaction components, this time can be significantly shortened but should be done in a systematic manner since the optimal extension time can be polymerase and sequence specific. In addition to these cycling conditions, it is often desirable to place a single denaturation step of three to five minutes at 94°C or 95°C at the beginning of the reaction and a final extension step of a few minutes at 72°C.

Table 1. Details of published primers used for PCR diagnosis of *Enterocytozoonhepatopenaei*

Primer name	Primer sequence (5' to 3')	Amolicon size (bp)	Target gene	Thermal cycling condition
MF1	CCGGAGAGGGAGCCTGAGA	951	SSU rRNA	Denaturation at 94°C, 3 min 35 cycles of -Denaturation at 94°C, 30 sec - Annealing at 55°C, 30 sec - Extension at 72°C, 90 sec Final extension at 72°C, 5 min
MR1	GACGGGCGGTGTGTACAAA			
ENF779	CAGCAGGCGCGAAAATTGTCCA	779	SSU rRNA	Denaturation at 94°C, 3 min 35 cycles of - Denaturation at 94°C, 20 sec - Annealing at 58°C, 20 sec - Extension at 72°C, 45 sec Final extension at 72°C, 5 min
ENR779	AAGAGATATTGTATTGCGCTTGCTG			
ENF176	CAA CGCGGGAAAACTTACCA	176	SSU rRNA	Denaturation at 94°C, 3 min 35 cycles of - Denaturation at 94°C, 20 sec - Annealing at 64°C, 20 sec - Extension at 72°C, 20sec Final extension at 72°C, 5 min
ENR176	ACCTGTTATTGCCTTCTCCCTCC			
EHP-510F	GCCTGAGAGATGGCTCCCACGT	510	SSU rRNA	Denaturation at 94°C, 3 min 35 cycles of - Denaturation at 94°C, 30 sec - Annealing at 60°C, 30 sec - Extension at 72°C, 30sec Final extension at 72°C, 5 min.
EHP-510R	GCGTACTATCCCCAGAGCCCGA			
SWP_1F	TTGCAGAGTGTTGTTAAGGGTTT	514	Spore wall protein	Denaturation 95°C, 5 min 30 cycles of - Denaturation at 95°C, 30 sec - Annealing 58°C, 30 sec - Extension 68°C, 45 sec Final 68°C, 5 min
SWP_1R	CACGATGTGTCTTTGCAATTTTC			
SWP_2F	TTGGCGGCACAATTCTCAAACA	148	Spore wall protein	Denaturation 95°C, 5 min 20 cycles of - Denaturation 95°C, 30 sec - Annealing 64°C, 30 sec - Extension 68°C, 20 sec Final 68°C, 5 min
SWP_2R	GCTGTTTGTCTCCAACTGTATTTGA			

Step 4: Validating the Reaction

Once PCR reaction has run, there are two ways of determining success or failure. The first is to simply take some of the final reaction and run it out on an agarose gel with an appropriate molecular weight marker to make sure that the reaction was successful and if the amplified product is the expected size relative to the maker. The ultimate validation of a PCR reaction is to directly sequence the amplicon. This is often a choice that is not readily available since not everyone has access to a DNA sequencer nor will they have either the time or the funds to carry out such an analysis.

Based on the size of the amplifiedproduct, 0.8 to 2% agarose gels are prepared either in1x Tris-Acetate-EDTA buffer (1 litre 50x TAE–242 g Tris base, 55 ml Glacial acetic acid and 37.2 gEDTA, pH 8) or 0.5 x Tris Boric acid EDTA buffer (1 litre 50x TBE – Tris base540 g, Boric acid 275 g and EDTA 18.5g,pH 8.0). Ethidium bromide is added to the molten agarose (0.5 µg/ml final concentration) when temperature reached 45-50^{o}C and then poured into the base. Once the gels are solidified, it is submerged in the tank with the samebuffer. A total volume of 5-10 µl amplified product is directly added to each well. Use 6x gel loading dye (for 100ml – 30mg Bromophenol blue, 30 mg Xylene cyanol, 12 ml of 0.5M EDTA pH8, 1ml of 1M Tris-Hcl pH8, 27 ml of distilled water and 60 ml of sterile glycerol) in case of ordinary master mix preparations. A molecular weight marker is also loaded to the gel to verifythe size of the amplified product. After loading, the tank is connected to a power pack and electrophoresis is carried out at voltage of 80-120. Continue the electrophoresis until the dyemigrates to the appropriate distance in thegel.

Observation and documentation

The gel is finally put in a gel-doc forcomplete analysis or on a UV transilluminator for visualization. Thepositive result is read in the form of aband at the right position in the gel. Absence of band indicates negativereaction or absence of the parasite, EHP. Presence ofband in the positive control and absenceof band in the negative control indicatesabsence of technical error orcontamination.

Fig. 1. Agarose gel electrophoresis showing detection of EHP by first step PCR
Lane M: 100bp Marker
Lane S:Sample (Negative)
Lane N:Negative control
Lane P:Positive Control
(Expected amplicon size: 779 bp)

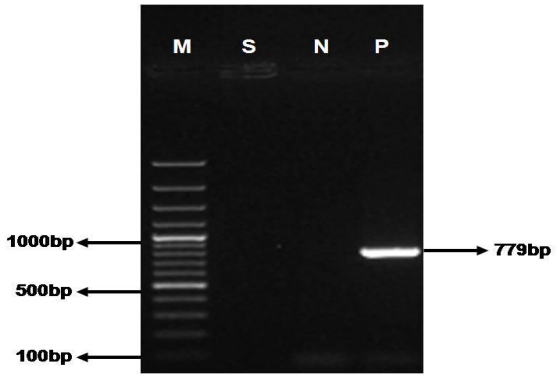
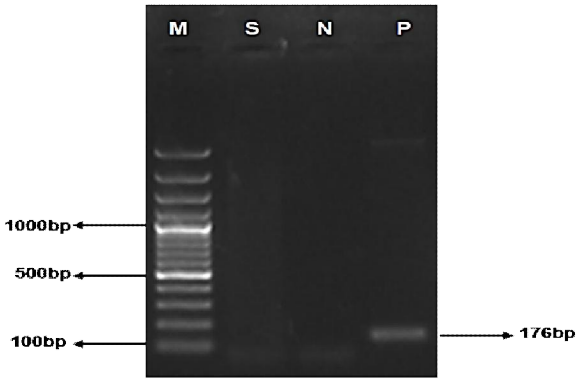


Fig. 2. Agarose gel electrophoresis showing detection of EHP by nested PCR
Lane M: 100bp Marker
Lane S:Sample (Negative)
Lane N:Negative control
Lane P:Positive Control
(Expected amplicon size: 176 bp)



Record maintenance

It is necessary to maintain a recordregarding the results of each sampling.This will help to interpret the overallsituation over a period of time.

Conclusions

EHP infection can be detected by demonstrating spores (1.1 ± 0.2 by 0.6-0.7 ± 0.1 µm) in light microscopy of stained hepatopancreas tissue smear, hepatopancreas tissue sections and faecal samples. In molecular methods, EHP can be detected by polymerase chain reaction, using extracted DNA from hepatopancreas tissue, faeces and whole Post Larva. Out of the available PCR protocols, PCR assay based on spore wall protein is highly specific. It can be used for screening the environment samples such as water, feed, faecal threads, carriers without any cross amplification. And also screening will be more specific in the case of non-lethal screening of shrimp brooders. Other molecular methods such as *insitu*-hybridisation, real time PCR and LAMP-nanogold method have also been described for the detection of EHP infection. Light microscopy may not detect low level of infection, resolving the spore also required a trained eye and unsuitable for non-destructive screening of shrimp faecal threads. Molecular methods such as Nested PCR, qPCR and LAMP may be the choice for EHP detection.

References

1. Itsathitphaisarn, O., Jaroenlak, P., Sanguanrut, P., Salachan, P.V., Wiredu-Boakye, D., Williams, B.A.P., Stantiford, G.D., Flegel, T.W. and Sritunyalucksana, K. (2016). A new and improved PCR detection method for *Enterocytozoonhepatopenaei* (EHP) based on a gene encoding a spore wall protein. Accessedonline http://www.enaca.org/modules/library/publication.php?publication_id=1177&title=improved-pcr-detection-of-ehpon 06.06.2016.
2. NACA (2015). Hepatopancreaticmicrosporidiosis caused by*Enterocytozoonhepatopenaei* (EHP)disease card (2015). Network ofAquaculture Centres in Asia-Pacific (NACA), Bangkok, Thailand. Accessed online http://www.enaca.org/publications/health/disease-cards/ehp-disease-card-2015.pdfon 04.06.2016.
3. Liu, T., Yang, B., Liu, S., Wan, X., Wang, X., Huang, J., 2014. PCR detection and studies on the prevalence of hepatopancreatic parvovirus (HPV). Progress in Fishery Sciences, Issue 4:66-70 (In Chinese with English abstract)
4. Suebsing, R., Prombun, P., Srisala, J., Kiatpathomchai, W., 2013. Loop-mediated isothermal amplification combined with colorimetric nanogold for detection of the microsporidian Enterocytozoon hepatopenaei in penaeid shrimp. Journal of Applied Microbiology. 114 1254-1263.
5. Tang, K.F.J., Pantoja, C.R., Redman, R.M., Han, J.E., Tran, L.H. and Lightner, D.V. (2015). Development of *in situ* hybridization and PCR assays for the detection of *Enterocytozoonhepatopenaei*(EHP), a microsporidian parasite infecting penaeid shrimp. *J. Invertebr. Pathol*. 130, 37-41.
6. Tangprasittipap, A., Srisala, J., Chouwdee, S., Somboon, M., Chuchird, N., Limsuwan, C., Srisuvan, T., Flegel, T.W. and Sritunyalucksana, K. (2013). The microsporidian*Enterocytozoonhepatopenaei* is not the cause of white feces syndrome in whiteleg shrimp *Penaeus*(*Litopenaeus) vannamei.BMC Vet. Res*., 9, 139-148.
7. Tourtip, S., Wongtripop, S., Stentiford, G.D., Bateman, K.S., Sriurairatana, S., Chavadej, J., Sritunyalucksana, K. and Withyachumnarnkul, B. (2009). *Enterocytozoonhepatopenaei*sp. Nov. (Microsporida: Enterocytozoonidae), a parasite of the black tiger shrimp *Penaeus monodon* (Decapoda: Penaeidae): Fine structure and phylogenetic relationships. *J. Invertebr. Pathol.,* 102, 21-29.

Real Time PCR and its applications in detection of shrimp pathogens.

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Introduction

Diseases caused by pathogens has been one of the major problems faced by the aquaculture industry. Diagnostic methods for the causative agents of these diseases include morphological pathology, wet mount preparations, histopathology, traditional microbiology, bioassay methods, electron microscopy, serological techniques and the application of PCR and its variants. The choice of diagnostic technique differs based on the type of disease, agent, economic feasibility etc.Diagnostic methods play two significant roles in aquatic animal health management and disease control. Some techniques are used to screen healthy animals (surveillance studies); and others are used to determine the cause of unfavourable health (diagnosis). Diagnostic methods are divided, depending on the technique used as presumptive and confirmative methods. Molecular methods, such as one-step and nested PCR/RT-PCR (Polymerase chain reaction/reverse transcriptase PCR) and real-time PCR, are now a days commonly used by shrimp disease diagnostic laboratories for surveillance and confirmatory diagnosis.

Real-time PCR

Real-time PCR methods have the advantages of speed, specificity and sensitivity than normal PCR and other traditional methods of Detection. The application of PCR is unique in identifying diseases/ detection of pathogen that it helps in detection of pathogen even before appearance of visible signs of disease. PCR helps in diagnosis where similar clinical symptoms are observed and will require more detailed study by histology or other methods.Added advantage the whole process by PCR takes only few hours to complete and the presence of suspected agents can be identified. PCR has completely revolutionized the detection of RNA and DNA. Traditional PCR has advanced from detection at the end-point of the reaction to detection while the reaction is occurring.

Principle of Real-Time PCR

Real-time detection of PCR products is made possible by including in the reaction a fluorescent molecule that reports an increase in the amount of DNA with a proportional increase in fluorescent signal. The fluorescent chemistries employed for this purpose include DNA-binding dyes and fluorescently labelled sequence specific primers or probes. Specialized thermal cyclers equipped with fluorescence detection modules are used to monitor the fluorescence as amplification occurs. The measured fluorescence reflects the amount of amplified product in each cycle.

The principle of the real-time detection is based on the fluorogenic 5'nuclease assay. During the PCR reaction, the DNA polymerase cleaves the probe at the 5' end and separates the reporter dye from the quencher dye only when the probe hybridizes to the target DNA. This cleavage results in the fluorescent signal generated by the cleaved reporter dye, which is monitored real-time by the PCR detection system. The PCR cycle at which an increase in the fluorescence signal is detected initially (Ct) is proportional to the amount of the specific PCR product. Monitoring the fluorescence intensities during Real Time allows the detection of the accumulating product without having to re-open the reaction tube after the amplification.

Non-specific detection: Real-time PCR with double-stranded DNA-binding dyes as reporters

A DNA-binding dye binds to all double-stranded (ds) DNA in PCR, causing fluorescence of the dye. An increase in DNA product during PCR therefore leads to an increase in fluorescence intensity measured at each cycle. However, dsDNA dyes such as SYBR Green will bind to all dsDNA PCR products, including nonspecific PCR products (such as Primer

dimer). This can potentially interfere with, or prevent, accurate monitoring of the intended target sequence.

In real-time PCR with dsDNA dyes such as SYBR green, the reaction is prepared as usual, with the addition of fluorescent dsDNA dye. Then the reaction is run in a real-time PCR instrument, and after each cycle, the intensity of fluorescence is measured with a detector; the dye only fluoresces when bound to the dsDNA (i.e., the PCR product). This method has the advantage of only needing a pair of primers to carry out the amplification, which keeps costs down; however, only one target sequence can be monitored in a tube.

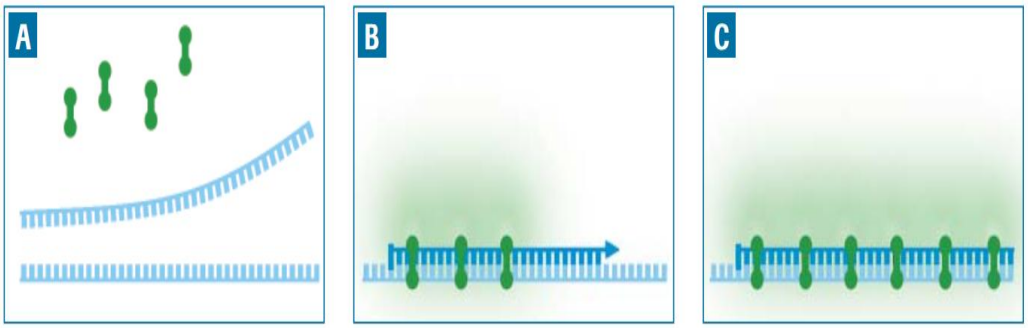


Figure: PCR in the presence of SYBR Green I.

SYBR Green I dye only fluoresces when it is bound to double-stranded DNA (dsDNA) and excited by blue light. SYBR Green I do not bind to single-stranded DNA, so fluorescence is minimal during denaturation. As dsDNA forms (panel A) and is synthesized (panel B), SYBR Green I binds the dsDNA and the fluorescent signal from the bound SYBR Green I (green light) increases. At the end of elongation (panel C), all DNA is double-stranded; the maximum amount of SYBR Green I is bound and the fluorescent signal is at its maximum for that PCR cycle. Therefore, the fluorescent signals from SYBR Green I are measured at the end of each elongation phase.

Specific detection: fluorescent reporter probe method

Fluorescent reporter probes detect only the DNA containing the sequence complementary to the probe; therefore, use of the reporter probe significantly increases specificity, and enables performing the technique even in the presence of other dsDNA. Using different-coloured labels, fluorescent probes can be used in multiplex assays for monitoring several target sequences in the same tube. The specificity of fluorescent reporter probes also prevents interference of measurements caused by primer dimers, which are undesirable potential by-products in PCR. However, fluorescent reporter probes do not prevent the inhibitory effect of the primer dimers, which may depress accumulation of the desired products in the reaction.

Hydrolysis probe assays, conventionally called TaqMan® assays, can technically be described as homogenous 5´ nuclease assays, since a single 3´ non-extendable hydrolysis probe, which is cleaved during PCR amplification, is used to detect the accumulation of a specific target DNA sequence. This single probe contains two labels, a fluorescence reporter and a fluorescence quencher, in close proximity to each other. When the probe is intact, the quencher dye is close enough to the reporter dye to suppress the reporter fluorescent signal (fluorescence quenching takes place via FRET). During PCR, the 5´ nuclease activity of the polymerase cleaves the hydrolysis probe, separating the reporter and quencher. In the cleaved probe, the reporter is no longer quenched and can emit a fluorescence signal when excited.

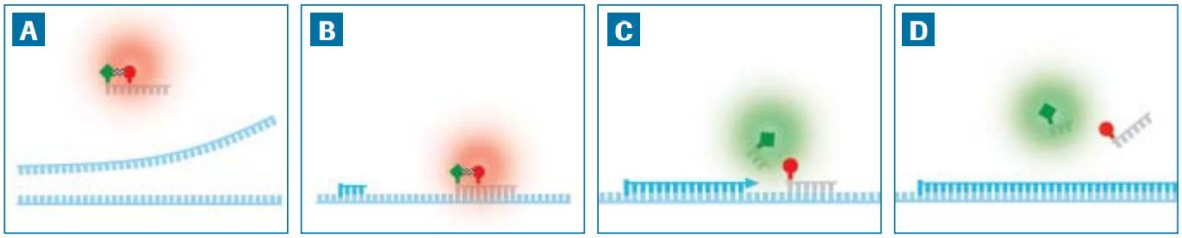
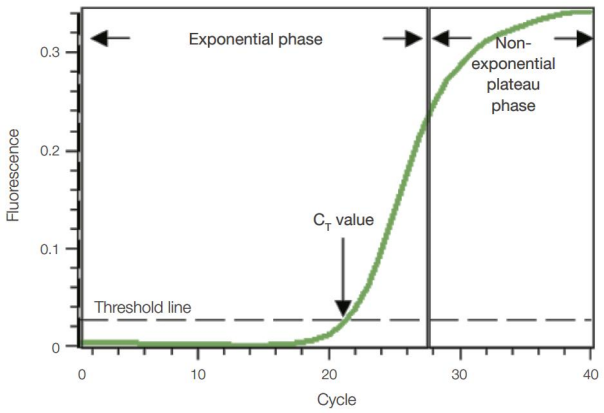


Figure: Schematic diagram of the Hydrolysis Probes format.

Panels A-D show the behaviour of hydrolysis probes during PCR. The probe carries two fluorescent dyes in close proximity, one of which (quencher) quenches the fluorescence from the other (reporter) as long as the probe is intact. In the denaturation phase (panel A), the strands of the target DNA separate as the temperature increases. During the annealing phase (panel B), primers and probes specifically anneal to the target sequence (the hydrolysis probe is phosphorylated at the 3' end, so it cannot be extended). As the DNA polymerase extends the primer, the 5' nuclease activity of the enzyme will cleave the probe (panel C), allowing the reporter dye to emit green fluorescence. The probe fragments are then displaced from the target and polymerization of the new amplicon continues (panel D). The DNA polymerase will separate the reporter and quencher only if the probe has hybridized to the target. Accumulation of PCR products is detected directly by monitoring the increase in green fluorescence from the reporter dye. The fluorescent signal of the reporter dye is measured at the end of each elongation phase (panel C).



In this plot, the PCR cycle number is shown on the x-axis, and the fluorescence from the amplification reaction, which is proportional to the amount of amplified product in the tube, is shown on the y-axis. The amplification plot shows two phases, an exponential phase followed by a non-exponential plateau phase. During the exponential phase, the amount of PCR product approximately doubles in each cycle. As the reaction proceeds, however, reaction components are consumed, and ultimately one or more of the components becomes limiting. At this point, the reaction slows and enters the plateau phase (cycles 28–40 in Figure).

Initially, fluorescence remains at background levels, and increases in fluorescence are not detectable (cycles 1–18 in Figure) even though product accumulates exponentially. Eventually, enough amplified product accumulates to yield a detectable fluorescent signal. The cycle number at which this occurs is called the threshold cycle, or CT. Since the CT value is measured in the exponential phase when reagents are not limited, real-time qPCR can be used to reliably and accurately calculate the initial amount of template present in the reaction. The CT of a reaction is determined mainly by the amount of template present at the start of the amplification reaction. If a large amount of template is present at the start of the reaction, relatively few amplification cycles will be required to accumulate enough products to give a fluorescent signal above background. Thus, the reaction will have a low, or early, CT. In contrast, if a small amount of template is present at the start of the reaction, more amplification cycles will be required for the fluorescent signal to rise above background. Thus, the reaction will have a high, or late, CT. This relationship forms the basis for the quantitative aspect of real-time PCR.

Quantitation

Choose correct wavelength of fluorophores under the instruction of Real-Time machine manufacturers.

- Key in the quantities of standard for pathogen
- Choose the correct flurophore to quantify the viral amount. Define the threshold. And then determine the threshold cycle number (Ct) of samples and standards.
- Make the calibration curve, log (quantity) vs. Ct, by at least 4 standards. Check the R square (R2) of the calibration curve to evaluate the linear correlation. The qualified range of R square is between 0.95 to 1. If not, try to re-define the threshold.
- Interpolate the Ct of each sample into the calibration curve to get its log (quantity), and then calculate back to its original quantity.
- The real time system software automatically sets threshold and gives the CT (cycle threshold) value for positive controls and samples.
- When there is a detectable CT value for the pathogen fluorophore, the sample will be treated as positive.
- Those samples that exhibit a CT Value of 35 or less treated as a positive
- CT value above 35 or if not determined the sample is considered as negative.
- The NTC reactions should not exhibit the presence of amplified DNA by fluorescence growth curves that cross the threshold. If a false positive occurs with NTC reactions, sample contamination may have occurred. Invalidate the run and repeat the assay with stricter adherence to the procedure guidelines.
- PTC reactions should produce a positive result with each reaction as demonstrated by fluorescence growth curves that cross the threshold. If expected positive reactivity is not achieved, invalidate the run and repeat the assay with stricter adherence to procedure guidelines.
- Determine the cause of failed PTC / positive NTC reactivity and implement corrective actions.
- Do not use reagents that do not generate the expected result for PTC.
- The Extraction Control should notexhibit fluorescence growth curves that cross the threshold line.

REAL TIME PCR TESTS - POINTS TO BE CONSIDERED

Always Mix the Reagents Before Use

- The reagents contain dyes, nucleotides, and enzymes that may have settled while sitting in the freezer or refrigerator.
- Vortex the master mix before aliquoting into tubes to avoid uneven distribution of reagents between samples.

Use Pipettes Accurately

- Use appropriate pipettes for the reaction set up
- Use a low volume pipetting which may reduce the pipetting errors
- Calibrate the pipette at regular intervals depending upon the usage

Perform a Standard Curve for Every New Primer Pair

- PCR efficiency can be impacted by a number of factors. The best practice is to run a 5 point standard curve with 10 fold dilutions for every new primer pair and make sure you can get at least 90% PCR efficiency with control DNA.

Follow the Three Room Rule

- One of the biggest causes of contamination is from using the same pipettes for extraction or handling PCR products post-run for reaction set up.

- Even if aerosol resistant tips are used avoid using same pipettes. Buy a complete set of pipettes that are used for PCR set up and nothing else.
- The ideal set up is to have three rooms; one for RNA or DNA extractions, one for reaction set up and one for the real-time cycler.

Double Check the Conditions

- This is important before hitting start, make sure the machine has the correct run cycle/program for the desired test.
- Do not use too much template/ template with high concentration.
- Real-time PCR is sensitive enough that sometimes less template gives a more accurate measurement.
- Samples that cross the threshold below cycle 15 will fall into most instruments default baseline setting and this will cause a subtraction of fluorescence from the rest of the data.
- This can be remedied by adjusting the baseline setting, but if you are unfamiliar with your instrument, it may require a call to technical service to figure it out. Also, if there were any inhibitors in the sample from the purification step (guanidine salts or ethanol, for example) diluting the sample will eliminate their impact on the results and give you an accurate quantitation of the sample.
- The best approach for a new sample is to perform a standard curve- even just a 3 point dilution series- to see what concentration will give you a Ct that falls in your standard curve and is most accurate.

Shrimp Disease diagnosis using real time PCR is increasingly becoming common now a days, several commercial kits are available for pathogens such as AHPND, WSSV and EHP. Published methods for detection of shrimp pathogens by real time PCR are available in OIE manual.Validation and quality check of reagents are essential for performing real time PCR tests.

PCR Reporting and role of aqua health laboratories

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Laboratory tests have a significant impact on decision making and provide information to aid in disease prevention, diagnosis and treatment.Laboratory tests are an important part of the process of successful aquaculture operations. The laboratories should establish and implement the most cost efficient and cost effective service delivery systems while maintaining the highest quality standards employing best working practices and protocols.It is the responsibility of the diagnostic laboratories to ensure that the tests are performed accurately and report the results to the requesting farmers / stakeholders as soon as possible and with recommendations on implementable measures to manage aquatic animal health to obtain successful crop. The vertical and horizontal integration of aqua farmers and associated laboratories in aquatic animal health management systems provides a new opportunity to bring the expertise and perspective of the separate disciplines to the evaluation of laboratory tests. This article presents the perspectives on the laboratory tests meant for aquatic animal health management with special reference to PCR.

Expectations of aqua farmers from diagnostic laboratories

The aqua farmers approach diagnostic laboratories with a hope to diagnose disease or identify the cause of mortality or abnormality in his farm, with recommendations on remedial measures which he can follow in his farm to obtain a successful harvest. What the aqua farmers would expect from the diagnostic laboratories is personalised care and concern about the crop in his farm.

Role of laboratory tests in aquatic animal health management

	Screening	*Diagnosis*	*Prognosis*	*Treatment*	*Monitoring*
Main purpose	Early disease detection, Risk assessment, and Screening	Diagnosis of a specific condition	Determine disease severity and risk stratification	Appropriate treatment selection and response	Continuous monitoring to assess disease severity and progression
Impact	Disease prevention	Timely and appropriate management	Management and risk mitigation	Improved health outcomes and successful aquaculture operation	Successful aquaculture operation

Reporting format

Some of the essential background information is required to be recorded provided to the diagnostic laboratory. History of the events prior to disease outbreak and information on the clinical signs (appearance, colour change etc.), behaviours (feeding pattern, swimming pattern, active / lethargy etc) will help arriving at diagnosis. The report should have background information of the contact farmer, source of sample, its description and problem in the farm.The report should mention briefly the material and methods employed in conducting the assay with appropriate citation. The results obtained should be indicated with illustrations, figures of gels and tables. The

information on a) quality checks on the control samples, b) concentration of nucleic acid, c) quality or integrity of nucleic acid, and e) limitationof test. Finally, conclusion / inference should be provided explicitly after carefully analyzing the results. Based on analysis of results, technical advice and follow-up actions may be suggested.

Conclusion

Laboratory tests are not simply diagnostic tools. They have the potential to provide value for the aquatic animal health management in their prognostic, treatment selection, disease and therapy monitoring applications. While the objective should be overall performance of the aquaculture operation, the limitations of laboratory diagnostics are partly because of the challenges inherent in linking diagnostic testing to decision making at farm level and its downstream effects on treatment and farm outcomes. Test performance (often measured by sensitivity, specificity, or other metrics) is only a surrogate end point and the link between test results and their downstream effects need to be supported by evidence. The aqua health laboratories should ideally include long-term follow-up to capture all immediate and downstream consequences of testing, including impact on treatment and outcome of farming.

PCR laboratory Layout; equipment and reagents.

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1. Introduction

Polymerase chain reaction (PCR) is the most sensitive diagnostic method being widely followed over the world, including India. Though the assay is with such a high sensitivity and specificity, it is prone to give false results due to contamination in reaction mixture or poor quality of the reagent/plastic-wares used. Hence it is utmost important to design the PCR laboratory in such a way that cross-contamination and carriers of contaminants are avoided. The chemicals and plastic-wares used for the assay should be of good quality to produce good results. In this regard, we need to consider layout of the PCR laboratory at the beginning of laboratory designing process. In addition, should consider the quality of the materials used and their storage condition for prolonged efficacy. Safety of the user while handling some carcinogenic reagents is very important. All these factors determine success of a diagnostic laboratory. The parameters to be considered while designing PCR laboratory layout are described below.

2. PCR laboratory layout

PCR laboratory layout is same either the lab is to handle small, medium or large number of samples. In a standard diagnostic PCR laboratory, work flow can be divided in to three stages, pre-PCR, PCR and post-PCR. In general PCR assay need to handle raw samples, process it to extract DNA, these are performed in pre-PCR section. Reaction mix that should contain *Taq* DNA polymerase, reaction buffer, dNTPs, primers and DNA template is prepared for PCR. The mixture is placed in a thermal cycler for DNA amplification and this is carried out in PCR room. The PCR product is checked for the result by gel-electrophoresis and observed on a UV trans-illuminator or a gel-documentation unit in post-PCR chamber. To minimise cross-contamination and improve work efficiency, these three steps in a PCR assay should be divided into three independent chambers with no cross-flow. The micropipettes, equipment, gloves, lab-coat etc. should not be shifted from one section to the other. Partitioning of the work, equipment selection, storage place, bench-working place etc. need to be judiciously planned.

(i) Pre-PCR area: In this section, the samples are received and aesthetically processed inside a laminar air flow/ biosafety cabinet/ sterile hood. A portion of the sample is used for nucleic acid extraction, as explained earlier chapters and the remaining part is stored in a -20°C freezer or -80°C freezer depending on the sample requirement or facility available. All these processes should be conducted by wearing a pair of gloves and clean lab-coat. A tape water point should be available for washing and cleaning purpose. DNA extracted is used for PCR in PCR room however it should be stored in -20°C freezer.

Essential materials needed:Dissection sets, gloves, tissue paper, containers or tubes for processing and storing samples and racks to hold the tubes, glass slides and cover slips for microscopic observation. Cryo-box to store DNA and an ice bucket should exclusively be available for pre-PCR chamber. Reagents for tissue processing and DNA extraction are also need.

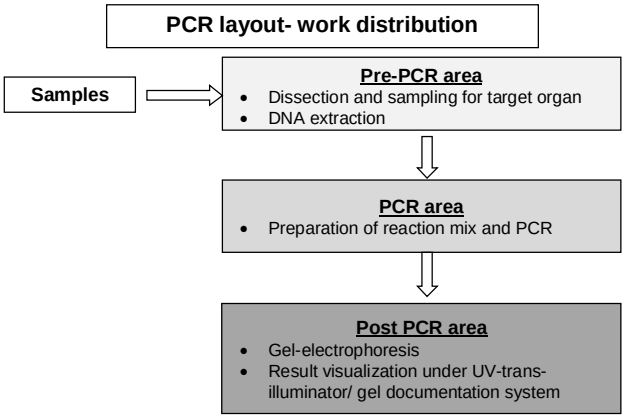
(ii) PCR area:A thermocycler is heart of this chamber and amplification of DNA is done in it. DNA prepared in pre-PCR chamber is mixed with other reagents for PCR, in a PCR tube (0.2 ml tube) inside a PCR work-station. PCR workstation can be sterilized by UV and is a closed cabinet hence cross contamination can be avoided from aerosols. PCR mixture should be prepared on ice. For this an ice bucket or mini-cool box (4°C) can be used. *Taq* polymerase and other contents for PCR are

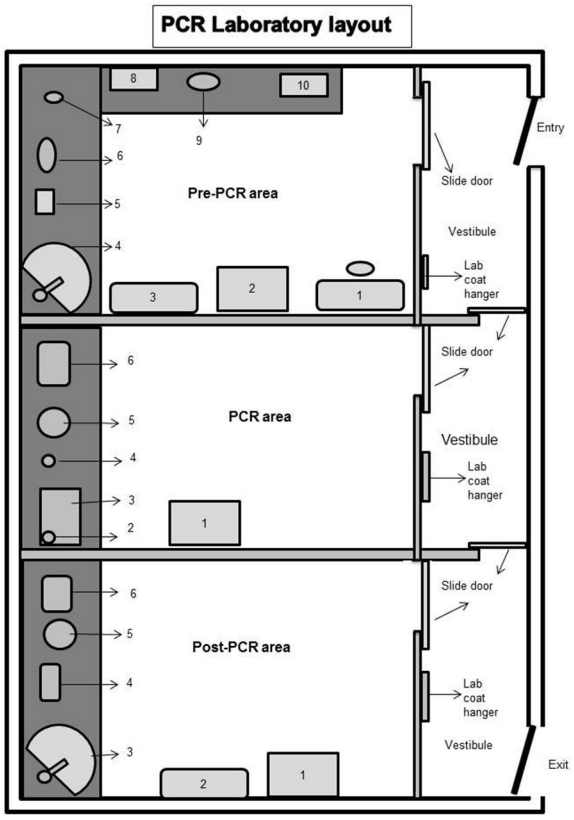
temperature sensitive hence should be maintained in a cool box while using. The enzyme should be stored at -20°C or as directed by the manufacturer. The room should be air-conditioned (25°C room temperature) for longer life and better performance of thermal cycler.

Essential materials needed: An ice bucket, mini cooler, rack for 0.2 ml tubes, PCR reagents.

(iii) Post-PCR area: Here the DNA amplified in thermal cycler will be checked for result. Gel electrophoresis will be performed here for result observation and the result will be read in a UV-trans-illuminator or gel-documentation system. Ethidium bromide is added to agarose gel before pouring on the gel casting tray that will help in visualizing the PCR product band. Ethidium bromide is highly carcinogenic and mutagenic. Hence it should be handled with utmost care so that no direct contact with the chemical should establish. Always use latex gloves while working in post-PCR. Ethidium bromide (<0.1%) should be dissolved in agarose gel when temperature reaches below 50°C, so that chances of inhalation of ethidium bromide through vapour is prevented. The gel and tips used should be discarded carefully in to a biohazard box. It should be incinerated periodically/ treated as chemical waste so that ethidium bromide does not reach environment. Once PCR product runs in gel, remaining part of it can be stored in a refrigerator for future reference if needed.

Essential materials needed: Tris-base acetic acid and EDTA (TAE) buffer or Tris Borate EDTA (TBE) buffer, DNA ladder (100 bp and 1k bp), ethidium bromide, gloves, tissue paper etc.





Pre-PCR area

1. Laminar air flow/ biosafety cabinet
2. Freezer
3. Ice flaker
4. Sink
5. Dry bath/thermos-shaker
6. Microscope
7. Micro-pipette stand
8. Tissue homogeniser
9. Vortex
10. Refrigerated centrifuge

PCR area

1. Freezer
2. Micro pipette stand
3. PCR work station
4. Vortex
5. Spin
6. Thermal cycler

Post-PCR area

1. Freezer/ Refrigerator
2. Gel documentation system/ UV trans-illuminator
3. Sink
4. Gel electrophoresis apparatus
5. Analytical balance
6. Microwave oven

Equipment

Quality of equipment and their maintenance plays crucial role in success of a PCR laboratory. Following equipment are needed to run a professional PCR based diagnostic laboratory. Equipment of similar specification is available from different manufacturers and vendors at varying price. A serious background study is needed at the time of purchase on the quality of the equipment, service rendered by supplier and budget available.

Section	Equipment
Pre-PCR	Micropipettes with tips and racks of different capacities
	Laminar air flow/ biosafety cabinet
	Refrigerated centrifuge for 2 ml tube capacity with 12000g force
	Microscope
	PCR workstation
	Vortex
	Refrigerator
	Ice flaker
	20°C freezer -80°C freezer (optional)
	Tissue homogeniser (optional, will ease DNA extraction process),
	Autoclave
PCR	micropipettes with tips and racks of different capacities
	PCR workstation
	vortex
	refrigerator
	-20°C freezer
	mini-spin
Post-PCR	Electrophoresis apparatus (Power pack, casting tray, and comb)
	Microwave oven
	Analytical balance
	Refrigerator
	Micro-pipette 10 µl capacity

3. Maintenance and management of laboratory, equipment and reducing cross-contamination.

In a successful operation of a PCR laboratory, proper maintenance of the laboratory equipment, work bench, premises are essential. The following points may be considered for ideal performance of the laboratory

- The working place once handled with a set of sample should be cleaned thoroughly and disinfected with 70% alcohol.
- Do not shift any equipment, reagents or consumables meant for one section to another one. It will cause cross contamination and will effect false positive results.
- All the high precision and sensitive equipment such as micro-pipette, microscope, laminar air flow, autoclave, tissue homogeniser, refrigerated centrifuge, thermal cycler, PCR work station and analytical balance should be handled carefully and calibrated regularly to keep up its efficiency.
- Micro-pipette is highly sensitive to pressure, hence should be handled carefully from falling down. Forced tip lifting will damage calibration and tip of the pipette, will increase pipetting error by varied volume of the reagents and sample.
- A thermocycler contains thermal block in which temperature is ramped up and down in very short intervals to carryout DNA amplification. This is controlled by Peltier-effect system. The system is very sensitive and it is ideal to maintain cool room temperature of 25°C for better performance and longer life span of the equipment.
- Laminar air flow and PCR workstation should be serviced regularly to clean their filters.
- Refrigerators should be cleaned regularly to remove frost.
- Refrigerated centrifuge should be operated after proper balancing the centrifuge tubes.
- Weekly cleaning of the laboratory should be carried-out to remove dust or other junks. It is ideal to regularly check the samples stored in the freezers to remove unwanted ones from stock-piling.

- Monitor availability of chemical reagents and enzymes to avoid any shortage or wastage. Use the chemicals before the expiry date commences.
- Handle carcinogenic and volatile reagents carefully, with proper care. Alcohol is highly combustible hence do not stock large volume in one place.
- Ethidium bromide is highly carcinogenic and mutagenic, handle with care. Use gloves for using and do not touch either ethidium bromide or the gel containing ethidium bromide in bare hand. While casting gel, mix ethidium bromide (<0.1% by volume) to the molten gel when its temperature is below 50°C. Once you handle this chemical discard the gloves and use fresh one. If same gloves used, ethidium bromide will stick to several places such as microwave oven, gel doc system or door knob. Dispose ethidium bromide as bio-medical waste.
- Maintain a record book for equipment with details of supplier and service person and service or calibration details.
- Maintain chemical inventory for efficient usage of chemicals and prevent wastage.
- Maintain log book for equipment.

Good Laboratory Practices for PCR Diagnostic Labs.

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Aim

- GLP embodies a set of principles that provides a framework within which laboratory test procedures are planned, performed, monitored, and reported.
- It is a quality system which intends to ensure through careful and accurate documentation, covering all aspects of test and of its environment, quality, integrity and reliability oftest data.

Objectives

- GLP makes sure that the data submitted are a true reflection of the results that are obtained during the test.
- GLP also makes sure that data is traceable.
- Promotes international acceptance of tests.
- Identifies principles for laboratory setup and workflow design that prevents lab contamination.
- Identifies examples of good/poor practices in lab setup and workflow.
- Given the current lab setup, identifies changes that must be made to prevent lab contamination.

Good Laboratory Practices

- Lab induction should be provided to all staff appointed before they get into the routine protocols.
- Conventional protection used in every lab such as use of gloves, lab coat and glasses when and where necessary have to be used with additional care to prevent cross contamination. Gloves must be changed as and when required.
- Implementation of Good Housekeeping practices that includes disposal of wastes and sharps appropriately.
- Making aliquot of Reagents to prevent cross contamination and to prevent loss of activity/quality.
- Avoid keepingtubes or reagents any longer than necessary.
- Label the reagentsand reaction tubes clearly.
- Usage of dedicated equipment/materials including paper, pen, lab coat and workbook for particular area in the laboratory.
- Cleaning/Disinfection of LAF/ work area with hypochlorite or with any other bleach before and after use.
- LAF can additionally be disinfectedby UV exposure.

SOP

- Written procedures for a laboratories program.
- They define how to carry out protocol-specified activities.
- Most often written in a chronological listing of action steps.
- They are written to explain how the procedures are supposed to work.
- This includes
  - ✓ Routine inspection, cleaning, maintenance, testing and calibration.
  - ✓ Actions to be taken in response to equipment failure.
  - ✓ Analytical methods
  - ✓ Definition of raw data

- ✓ Keeping records, reporting, storage, mixing, and retrieval of data

Laboratory Setup

- Maintain unidirectional flow of work
- Provide Separate areas for the entire laboratory operations that includes sample preparation , Nucleic acid extraction, amplification and Post PCR.
- Ideally each of these steps takes place in a dedicated room and each room must be provided with a dedicated set of equipment, reagents, pipettes, and disposables.
- A dedicated set oflab coats that are kept and used exclusively in the appropriate room.

Cultivating Good Laboratory Habits to Prevent Cross-Contamination

- Each person's actions introduce significant risks of contaminating the PCR samples .
- Therefore, preventing cross-contamination requires the cooperation of all laboratory personnel. Only if the all people in the laboratory follow good work habits, will the laboratory be free of contamination.
- Never be tempted to take shortcuts in a PCR procedure, or to ignore the precautions out-lined.
- Never reverse the direction of workflow (eg. by carrying amplified material into the DNA extraction room).
- The steps in the PCR workflow are always unidirectional, from DNA extraction to amplification/ gel documentation. This principles holds for working procedures as well as for reagents and consumables.

Developing Laboratory Work Guidelines: Assessing and Avoiding risks in PCR workflow

- In developing good laboratory habits, start by attempting to quantify the hazards and risks a worker may encounter during the entire experimental procedure.
- A contamination hazard may be defined as the introduction of contaminating nucleic acid from any possible source; risk is the possibility that the hazard will occur.
- The goal of a risk assessment is to carefully consider all hazards and risks associated with the whole process, so that the risk can be minimized and preventive actions can be implemented.
- Any change in the process(e.g., new lab personnel, new equipment, a new supplier of consumables or a change in assay design) requires a new risk assessment.
- For risk assessment, consider sample handling steps, materials(reagents and disposables) used, and storage of intermediate products such as extracted DNA.
- Once the risks has been identified and quantified, the laboratory can adapt strategies to minimize or avoid these risks.

Instrument Calibration

- Calibration of equipment used in the molecular diagnostic laboratory is essential to generate reliable and accurate test data/ results. This is a process necessary for all diagnostic laboratories.
- Data produced by "faulty" instruments may give the appearance of valid data.
- The frequency for calibration, re-validation and testing depends on the instrument and extent of its use in the laboratory.
- Whenever an instrument's performance is outside the "control limits" , Use of the instrument must be discontinued and immediately repaired.

Handling of reagents

- Reagents should be stored properly at appropriate temperature.

- Avoid frequent freeze/thawing.
- Aliquot from stock solution to make working solution.
- Use light sensitive reagents in amber bottle or tubes.
- Carefully handle the carcinogenic chemicals or corrosive reagents with safety precautions.

Positive Control preparation and handling

- FOR KIT METHOD
  - ✓ Dissolve the plasmid in tRNA
  - ✓ At least take 5 dilution of positive control
  - ✓ Avoid frequent freeze/thawing
  - ✓ Separately store the positive controls in its dedicated area at -20^{0}C
  - ✓ Spin down the control tubes before use
  - ✓ Avoid creating aerosol during the addition and suspending
  - ✓ Take care during discard of micro tips in the discard

Handling of Sample

- ✓ Take adequate care of the sample from receipt, storage, processing.
- ✓ Wear gloves while handling samples and change the gloves as and when required.
- ✓ Label and store sub-sample carefully to serve as backup for retest .
- ✓ Label working sample properly.
- ✓ Process sample in clean environment using sterile/disinfected implements.

Discard of wastes

- ✓ Have a plan and policy for proper discard of laboratory wastes.

General Laboratory Biosafety

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In view of rapid development, in both basic and applied research in the field of aquaculture and related disciplines, there are concerns with respect risks to research staff during laboratory operations and overall risks due to the waste generated during the research activities. All the general laboratory safety regulations outlined for any R&D lab also apply to the shared PCR laboratories as well. The purpose of this chapter is to provide specific guidance and protocols for the protection of the users of the shared PCR laboratories. The plan proposed here will help to optimize use of equipment, space and safety of the workers at large.

Biosafety requirements in shared PCR laboratories

1. Biosafety for research staff

1.1 Personal protection

1. Laboratory coveralls, gowns or uniforms and appropriate gloves must be worn for all procedures that may involve direct or accidental contact with hazardous chemicals and other potentially infectious materials
2. After use, gloves should be removed aseptically and hands must then be washed.
3. Personnel must wash their hands after handling infectious materials and animals, and before they leave the laboratory working areas.
4. Safety glasses, face shields (visors) or other protective devices must be worn when it is necessary to protect the eyes and face from splashes, impacting objects and sources of artificial ultraviolet radiation.
5. It is prohibited to wear protective laboratory clothing outside the laboratory, e.g. in canteens, coffee rooms, offices, libraries, staff rooms and toilets.
6. Open-toed footwear must not be worn in laboratories.
7. Eating, drinking, smoking, applying cosmetics and handling contact lenses is prohibited in the laboratory working areas.
8. Storing human foods or drinks anywhere in the laboratory working areas is prohibited.
9. Protective laboratory clothing that has been used in the laboratory must not be stored in the same lockers or cupboards as street clothing.

Procedures

1. Pipetting by mouth must be strictly forbidden.
2. Materials must not be placed in the mouth. Labels must not be licked.
3. All technical procedures should be performed in a way that minimizes the formation of aerosols and droplets.
4. The use of hypodermic needles and syringes should be limited. They must not be used as substitutes for pipetting devices or for any purpose other than injection or aspiration of fluids from laboratory animals.
5. All spills, accidents and overt or potential exposures to infectious materials must be reported to the laboratory supervisor. A written record of such accidents and incidents should be maintained.
6. A written procedure for the clean-up of all spills must be developed and followed.
7. Contaminated liquids must be decontaminated (chemically or physically) beforedischarge to the sanitary sewer. An effluent treatment system may be

required,depending on the risk assessment for the agent(s) being handled.

8. Written documents that are expected to be removed from the laboratory need to be protected from contamination while in the laboratory.

1.2 Guidelines for biosafety - laboratory operations

1. 'Learners' in the lab should be taught the 'dos and don'ts' of Lab practices before they start the work.
2. Laboratories should be clean and items arranged in an orderly manner, free from materials that are not pertinent to the work.
3. Work surfaces must be decontaminated after any spill of potentially dangerous material and at the end of the working day.
4. All contaminated materials, specimens and cultures must be decontaminated before disposal or cleaning for reuse.
5. The cupboards/cabinets in the laboratory should be labeled /categorized properly for easy availability/identification of materials (chemicals /glassware /plasticware/or any other items) and items should be replaced properly.
6. Laboratory items/furniture (glassware/equipments/fume hood/ balances) have to be used for the identified purpose only.
7. Beverages and other food items should never be served / consumed inside the lab.
8. Inflammable liquids should be kept in separate containers with red paint marking (danger mark) and should be stored in fire-proof cabinets.
9. Acids and volatile solvents and digestion of biological samples should be handled strictly under fume hood.
10. Every lab should have first aid box with emergency eye washer. Researchers/staff should use aprons whenever/wherever they handle hazardous materials.
11. Corrosive liquids should be handled properly using corrosive-resistant hand gloves.
12. Gas cylinders containing explosive gases should be kept outside the laboratories.

1.3 Guidelines for bio-safety- disposal of waste generated during research activities

Hazardous laboratory wastes need to be monitored and disposed properly. There is a need for bio-safety guidelines for proper disposal of waste generated in research activities. Materials for decontamination and disposal should be pre-sorted and placed in autoclavable plastic bags that are colour-coded- according to whether the contents are bio-degradable, non-biodegradable or sharps. Materials to be autoclaved or incinerated should be separated. No pre-cleaning should be attempted of any contaminated materials before it is decontaminated by autoclaving or disinfection.The procedure for handling/disposal for contaminated materials/wastes along with separation system and categories to be followed are listed as below:

1. Non-contaminated (non-infectious) waste can be disposed of as general waste.
2. Contaminated "sharps" – hypodermic needles, scalpels, knives and broken glass; these should always be collected in puncture-proof containers fitted with covers and treated as infectious. Disposable syringes, used alone or with needles, should be placed in sharps disposal containers and incinerated, with prior autoclaving if required. Sharps disposal containers must be disposed off before filled to the capacity. Sharps disposal containers must not be discarded in landfills.
3. Contaminated material should be decontaminated by autoclaving and thereafter washing and reuse or recycling should be done (such as glass petri-plates,

test tubes, glass wares used for microbiological work.

4. Biodegradable contaminated material (agar, culture broth, tissue culture slants, cotton and swabs, protein and fats) should be segregated in colour coded bags and decontaminated by autoclaving before disposal.
5. Non-biodegradable (plastic tubes and tips,organic recalcitrant chemicals, gloves, gel wastes, glass, media tins and bottles, rubber, aluminium foils etc.) should be segregated in colour coded bags before decontamination and disposal.
6. The research division/section/unit heads should train personnel working in various labs to manage the waste generated as per these guidelines. Extra sensitization is required for those working with disease pathogens and molecular biology work.
7. Concentrated stock solution of toxic chemicals such as heavy metals, nitrogenous, sulfurous and phosphorous compounds, reagents used for routine analysis and ethidium bromide should not be disposed in the wash basin. They should be deactivated before disposal. To avoid the need for deactivation, minimum quantity of stock solution as per requirement should be prepared, preserved properly and also be used fully without any disposal. Some examples of hazardous chemicals and their deactivation procedures are listed in Table 1.
8. All lab workers should wear appropriate safety gears while handling waste.
9. Research workers should handle gel containing ethidium bromide only with gloves. Such gels should be biosorbed with activated charcoal before disposal. The gloves used for the molecular work, should never be exposed outside the lab or allowed to contaminate other areas.
10. Glassware / plasticware used for micro-organisms should properly be sterilized.
11. Hazards of microbial/other pathogen waste generated should properly be disposed by adopting good lab practices, for which persons involved should be trained.
12. Dead and post-experimental animals should be disposed of by incinerating them in pits covered with soil.
13. Based on the activity of hazardous waste, they can be identified as corrosive, inflammable, ignitable, reactive, toxic, infectious and radioactive. If such wastes are identified and their hazardous nature cannot be eliminated, special precautions as mentioned in the Table-1 need to be taken and if required separate guidelines would need to be developed.
14. Gloves (contaminated with ethidium bromide or other toxic substances) shouldnot be worn to answer the telephone or use other equipment in the lab.
15. Ethidium bromide solutions should be disposed of in the red waste containerlocated in the fume hood/staining bench of PCR lab. If hazardous waste other than ethidium bromide is put into these containers itshould be clearly marked on the label attached.
16. Ethidium bromide spills should be cleaned up immediately, using properprecautions (wear gloves, put material used to clean up in bio-hazardous wastebins, wipe contaminated area down well with ethanol or detergent).
17. Used gels and gel waste should be discarded in the bio- hazardous waste bin, which is located beneath the gel electrophoresis benches.
18. Used gels and gel waste should not be put down any of the sinks.
19. Packing and transportation must follow applicable national and/or internationalregulations.

1.4. Laboratory Rules for shared laboratory

Each laboratory should develop a system of regulations most suited for managing smooth and ambient work flow.

1. Users should be provided with their own bench space when possible. It is the responsibility of the user to keep their bench space clean. When bench space is shared users should be respectful of the other user's space.
4. Users are responsible for their own dishes and spills. Clean up is NOT the responsibility of the laboratory supervisors.
5. Dishes should NOT be left in the gel electrophoresis tank.
6. Broken or malfunctioning equipment should be reported to a laboratory supervisor IMMEDIATELY.
7. Instructions on how to use the thermocyclers, spectrophotometer, Imaging System and centrifuge are keptbeside them.
8. All users must make entry and sign in the logbook prior to use.
9. Pipettes labeled for PCR use should be used for PCR only. NO EXCEPTIONS.

Table 1- Disposal of chemical wastes in a PCR laboratory

Chemical	Method of deactivation
Ethidium bromide	Filter the ethidium bromide solution through charcoal filtration. Pour filtrate down the drain. Place charcoal filter in sealed bag and place in biohazardous waste box for incineration
Recombinant DNA	Deactivate recombinant DNA using DEPC or UV radiation or by autoclaving
Heavy metals	Minimum quantity of stock solution as per requirement should be prepared, preserved properly and also be used fully without any disposal because heavy metals cannot be biodegraded or eliminated
Nitrogenous, sulfurous and phosphorous compounds	Minimum quantity of stock solution as per requirement should be prepared, preserved properly and also be used fully without any disposal.
Reagents for routine analysis of chemical and biochemical parameters	Minimum quantity of reagents as per requirement should be prepared and should be used fully.
Volatile solvents and acids	Volatile solvents and acids should strictly be handled under fume hood. In the case of any leakage or spillage, all the doors and windows should be opened and the entry of persons should be restricted in the lab till complete escape of fumes. In the case of person having any accident with the spillage of acid, only water should be applied (please avoid the use of alkali) and should immediately be taken to nearby hospital. All the labs should have medical aids.

Guidelines for ethidium bromide waste management and disposal

Ethidium bromide is a compound used in many PCR laboratories for visualization of nucleic acid during gel electrophoresis applications. Ethidium bromide solution is incorporated into the electrophoresis gel as a dye for the DNA, RNA, or other molecules to be visualized. Ethidium bromide is mutagenic and moderately toxic and must be handled with care. For safety considerations, following practices are advised:

- The powder form is considered an irritant to the upper respiratory tract, eyes, and skin. Ethidium bromide is mutagenic; hence preparation of stock solutions and any operations capable of generating ethidium bromide dust or aerosols should be conducted in a fume hood to prevent inhalation. Nitrile gloves, a lab coat, and eye protection should be worn at all times, as with working with any hazardous material.

- When working with ethidium bromide, minimize the potential for spills. Where practical, purchase pre-mixed stock solutions from chemical manufacturers *in lieu* of preparing solutions. If solutions of ethidium bromide must be prepared, consider performing this process in a fume hood.
- Spills must be addressed immediately; for small spills (<20 ml) of concentrations up to 10 mg/ml; absorb with paper towel and then treat the area with 70-95% ethanol/isopropanol and wipe up-all to be collected as hazardous waste. Repeat as necessary (if still fluorescing). Final rinse with soap and water (paper towels for this step were discarded into regular trash). DO NOT clean ethidium bromide spills with bleach solutions.
- When an ultraviolet light source is used in work with ethidium bromide, added caution is required. As a general rule, avoid exposing unprotected skin and eyes to intense UV sources.
- Ethidium bromide waste should not be poured down the drain, or thrown in the trash, unless the waste has been deactivated or filtered. The following are the recommended disposal procedures for ethidium bromide:
- Dry the gels under the hood in an open container before bringing to the waste room for disposal. Dilute aqueous solutions containing <0.5 mg/ml ethidium bromide can be filtered or deactivated. Concentrated solutions (>0.5 mg/ml) should be collected for disposal as hazardous waste. Similarly, solutions that contain heavy metals, organics, cyanides or sulfides should be collected and disposed of as hazardous waste. Filtering the aqueous ethidium bromide waste solutions, free of other contaminants, through a bed of activated charcoal is a relatively simple and effective method for removal of ethidium bromide. The filtrate may then be poured down the drain.
- Autoclaving chemical waste creates exposure to the chemicals as they heat and volatilizes; hence should not be attempted.
- Consider switching to less-toxic alternatives to ethidium bromide (SYBR Safe DNA gel stain, for example) to reduce potential hazardous exposures in the lab.

Bibliography

1. Centers for Disease Control and Prevention (2011). Guidelines for Safe Work Practices in Human and Animal Medical Diagnostic Laboratories, MMWR 2011; U.S. Department of Health and Human Services, Atlanta, GA 30333, 61 (Suppl), p 102.
2. CPCSEA (2003). Guidelines For Laboratory Animal Facility, Committee for the Purpose of Control and Supervision on Experiments on Animals, N. 13/1, 3rd Seaward Road, valmiki Nagar, Thiruvanmiyur, Chennai, Tamil Nadu (India), p 38.
3. DBT (2011). Guidelines and Handbook for Institutional Biosafety Committees (IBSCs), Second edition, Ministry of Science & Technology & Biotech Consortium India Limited, New Delhi, p 120.
4. ICMR (2008). Guidelines for Good Clinical Laboratory Practices (GCLP). Indian Council of Medical Research, New Delhi, p 46.
5. WHO (2004). *Laboratory biosafety manual, Third edition,* World Health Organization, Geneva, p 178.

Note: This document is an extract prepared with focus on biosafety of laboratory professionals scoping the nature of work at CIBA with an intention to promote safe and good laboratory practices. Since the institute is not engaged on research related to radioisotopes or zoonotic pathogens, the specific guidelines on these aspects are not covered in this document. For more specific and detailed information on other related topics on biosafety guidelines, please refer the documents listed above.