# PROJECT DOCUMENT

## ACHIEVEMENTS FOR YEAR 2019

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| | | | **Project ID**: 201503001 |
| **Program Category** | ASEAN-SEAFDEC ASSP and FCG Mechanism | | |
| **Project Title** | Reinforcement and Optimization of Fish Health Management and the Effective Dissemination in the Southeast Asian Region | | |
| **Program Strategy No.** | II | **Total Duration** | 2015-2019 |
| **Lead Department** | Aquaculture Department (AQD) | **Lead Country** | None |
| **Donor/Sponsor** | Japanese Trust Fund (JTF) | **Total Donor Budget** | USD 383,107 |
| **Project Partner** | None | **Budget for 2019** | USD 65,510 |
| **Project Leader** | Koh-ichiro Mori / AQD | Project Participating Country(ies) | All Member Countries |

## PART I: OVERALL PROJECT DESCRIPTION

### 1. Brief Project Description:

This Project titled "Reinforcement and optimization of fish health management and the effective dissemination" was proposed to:

**1) Develop and accelerate rapid and effective fish and shrimp health management**
Preventive management strategies should be established to maintain the disease-free status for cultured stocks. Technology for early detection of these devastating viruses should be developed using optimized practical molecular diagnostic tools.

**2) Enhance efficacy of vaccine treatment in tropical cultured species**
Vaccines for NNV and WSSV have been developed. However, the efficacies of these treatments need further improvement. Technology of oral delivery of vaccine will be developed for the practical use in aquafarmers using carriers possessing immunomodulatory to enhance the overall immunogenicity of the vaccine.

**3) Establish protective measures against persistent and emerging parasitic diseases of tropical fish**
Avoidance of persistent and emerging parasitic diseases is of prime importance to secure sustainable production of food fish in the Southeast Asian Region. To avoid economic losses due to pressing problems attributed to parasitic infections in cultured fish, novel and practical prevention and control strategies should be established. Additionally, the species diversity of emerging parasites including their morphological characteristics and life cycle should be thoroughly investigated.

**4) Identify risk factors and develop protective measures against Early Mortality Syndrome (EMS)**
Based on the etiological agents together with identification of risk and protective factors, the protective measures will be developed against EMS. Guidelines to protect shrimp from EMS will be established.

**5) Extend & demonstrate technology to practitioners, officers, etc. of member countries**

Training programs will be implemented on specific topics based on the request from Member Countries, which necessitate the information dissemination on fish health management. In the first year, 2015, the 2-3 days course about EMS will be commenced in Myanmar.

## 2. Background and Justification:

The Aquaculture Department of the Southeast Asian Fisheries Development Center (SEAFDEC/AQD) initiated the Fish Disease Projects funded by the Government of Japan in response to numerous requests from various sectors for intensified research on fish health-related problems arising in the Southeast Asian region. Phase I (2000-2004) of the said projects focused on technologies to control diseases through timely and accurate recognition, sound diagnostic capabilities, and control measures for various diseases. Phase II (2005-2009) focused on disease surveillance activities based on the results of the earlier program. Thereafter, the importance of accelerating the delivery of information awareness among aquafarmers and the establishment of disease prevention methods emerged after reviewing the outcomes of the previous two project phases. To attain the above targets, Phase III (2010-2014) with the main topic "Accelerating awareness and capacity building in Southeast Asia" has been focusing on the greater dissemination of knowledge relevant to fish heath management, especially to the SEAFDEC Member Countries (MCs) whose capacities still need to be developed and improved. At the same time, innovative researches and technology development have been also implemented.

An integrated fish-health-care system expected to be established through the Phase III project aimed to ensure a holistic approach toward "healthy and wholesome" aquaculture practices enabling a stable supply of safe aquaculture products. The concept of the holistic approach was one of the six themes under Sustainable Aquaculture during the ASEAN-SEAFDEC Conference on Sustainable Fisheries for Food Security in the New Millennium: "Fish for the People" that was held in Bangkok in November 2001, and later in June 2011.

In practice, irrespective of the correct direction of the approach, however, there are still high needs for information dissemination and technology transfer in MCs, especially in lesser developing countries in terms of fish health management. In addition, serious issues relevant to the fish health management to be overcome have been often emerging, battering and disconcerting aqua farmers as shown in emerging diseases like Early Mortality Syndrome (EMS) hindering the wholesome development of aquaculture in our region. Realizing that the global market has become more stringent for exporting countries like the Southeast Asian countries, it is highly recommended to effectively disseminate the useful information such as precaution of potential diseases outbreak and recommendation of appropriate fish health management to local government unit officers, aquafarmers, traders, etc. promptly and appropriately. Urging better understanding on the risks, impacts and management problems related to diseases is very important because health management practices significantly affect product quality as well as quantity, and thus link with the economic stability for aquafarmers and fisheries community development.

## 3. Project Overall Objectives, Outcomes, Outputs, Indicators and Activities:

### 3.1 Objectives, Outcome and Output of the Project

| Objective | Outcomes | Outputs | Activities |
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| **Objective 1:** To develop and accelerate rapid and effective fish and shrimp | **Outcome 1:** Development and acceleration of rapid and | **Output 1:** Establishment of the early and effective intervention | **Activity 1: Development and acceleration of rapid and effective fish and shrimp health management**<br>➢ Determination of threshold infection levels for WSSV and other pathogen |

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| health management | effective fish and shrimp health management. | strategies through strict monitoring of the health status on cultured shrimp and fish by the farmers. | such as $VP_{AHPND}$<br>➢ Development of optimized q-PCR protocols for the detection of WSSV and $VP_{AHPND}$ |
| **Objective 2:** To enhance efficacy of vaccine treatment in tropical cultured species | **Outcome 2:** Enhancement of efficacy of vaccine treatment in tropical cultured species. | **Output 2:** Development of the practical method of delivering vaccine to fish with increased efficacy thereby preventing unwarranted outbreaks of VNN in hatcheries and grow-out culture systems.<br><br>Development of the method of delivering vaccine to shrimp with increased efficacy and prevention of white spot disease by interfering with its replication in the host. | **Activity 2: Enhancement of efficacy of vaccine treatment in tropical cultured species**<br>➢ Evaluation of the field efficacy of the inactivated NNV vaccine against natural or experimental NNV infection in pompano (net cage, open sea) and grouper (earthen pond)<br>➢ Elucidation of the effect of primary sublethal NNV injection followed by lethal NNV injection in pompano in conjunction with the antibody production and conferment of protection (RPS)<br><br>➢ Evaluation of the efficacy of RNAi in protecting shrimp against WSSV infection<br>➢ Development of delivery scheme using a combination of the antiviral treatments<br>➢ Development of low-cost delivery method for the antiviral treatments: tank studies<br>➢ Field Efficacy Evaluation of the combined antiviral treatment |
| **Objective 3:** To establish protective measures against persistent and emerging parasitic diseases of tropical fish | **Outcome 3:** Establishment of protective measures against zoonotic diseases of fish. | **Output 3:** Establishment of practical and efficient protocol for the prevention and control of persistent and emerging fish parasites | **Activity 3: Establishment of protective measures against persistent and emerging parasitic diseases of tropical fish**<br>➢ Development of practical strategies that could be adopted by farmers to address the pressing problem on mass mortalities of net-caged and pond reared fishes attributed to persistent and emerging fish parasites |
| **Objective 4:** To identify risk factors and develop protective measures against Early Mortality Syndrome | **Outcome 4:** Development of protective measures against emerging diseases. | **Output 4:** Establishment of practical and effective methods for the prevention and control of EMS | **Activity 4: Epidemiology of the Early Mortality Syndrome (EMS)**<br>➢ Development of the protective measures against EMS, based on the etiological agents together with identification of risk and protective factors.<br>➢ Establishment of guidelines to protect shrimp from EMS. |

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| (EMS) /Acute Hepatopancreatic Necrotic Disease (AHPND) | | | |
| **Objective 5:** To extend & demonstrate technology to practitioners, officers, etc. of member countries | **Outcome5:** Technology extension and demonstration to practitioners, officers, etc. of Member Countries through training courses. | **Output5:** Effective and functional development of fish health management and guarantee for the sustainable development of aquafood production together with the poverty alleviation in Southeast Asian countries. | **Activity 5: Technology extension and demonstration**<br>➢ Implementation of training programs on specific topics based on the request from Member Countries, which necessitate the information dissemination on fish health management. |

### 3.2 Overall Scope/Description of Project

| Activity | Description |
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| **Activity 1**: Development and acceleration of rapid and effective fish and shrimp health management | Viral and bacterial diseases have caused major constraints in shrimp farming in most Asian countries and in the world. The continued occurrence of the most devastating viral disease, the white spot syndrome virus (WSSV), and other pathogens such as $VP_{AHPND}$ that cause acute hepatopancreatic necrosis disease (AHPND) necessitate the establishment of domesticated shrimp stocks that are free of these pathogens. Early detection of these devastating pathogens is the most efficient response to be able to implement immediate and appropriate interventions for the control of the spread of infection. Early detection of these devastating pathogens is the most efficient response to be able to implement immediate and appropriate interventions for the control of the spread of infection. Prompt diagnosis will give fish and shrimp farmers better health management of their stocks which will in turn minimize the losses due to diseases. Molecular-based techniques such as the use of polymerase chain reaction (PCR), quantitative polymerase chain reaction (q-PCR) and loop mediated isothermal amplification (LAMP)-based detection methods will be considered. Development and optimization of conventional and quantitative polymerase chain reaction-based detection methods will enable farmers to strictly monitor health status so that early and effective intervention strategies can be implemented. Adoption and development of LAMP offers a cheaper, more rapid and convenient detection method for existing and emerging shrimp and fish pathogens. These developed and optimized practical molecular diagnostic tools will be primarily adopted in Fish Health Diagnostic Laboratories. (Locations: Philippines and other Member Countries concerned) |

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| **Activity 2**: Enhancement of efficacy of vaccine treatment in tropical cultured species | **Sub-activity 2.1** Enhancement of vaccine efficacy for the prevention of viral nervous necrosis in high value marine fish. This study will pilot test the field efficacy of the previously developed inactivated NNV vaccine in pompano and groupers reared in floating net cages and earthen pond, respectively. Moreover, the feasibility of using a sublethal dose of NNV followed by subsequent exposure of these fish to a higher inoculum dose of the homologous NNV as a practical strategy to induce potent production of NNV-neutralizing antibodies and concomitant protection against experimental NNV challenge will be elucidated. Outputs of this study are expected to be used as practical strategies to combat unwarranted outbreaks of VNN in pompano, groupers, and other susceptible marine fish species particularly during the nursery or early phase of culture when these fish species are highly susceptible to the disease.<br><br>**Sub-activity 2.2** Application of adjuvants, carriers and RNAi technology to enhance the antiviral immune response of shrimp to WSSV<br>The objective of the study is to develop and adopt methods to enhance the efficacy of present vaccines for shrimp and other antiviral approaches such as RNAi. Similar to above, methods for vaccine production for WSSV will be adopted from JTF5 studies. The delivery vehicle will be based on the results of the previous vaccination study in shrimp. In addition, recent trends utilizing RNAi as an antiviral strategy in shrimp culture will be adopted. After the shrimp have been subjected to these antiviral treatments, the shrimp will be experimentally challenged based on established procedures. Efficacy of the vaccines/RNAi treatment will be evaluated based on RPS. The expected output from the study is a method of delivering vaccine to shrimp with increased efficacy and prevention of white spot disease by interfering with its replication in the host. (Location: Philippines and other Member Countries concerned) |
| **Activity 3**: Establishment of protective measures against persistent and emerging parasitic diseases of tropical fish | The primary goal of this study is to develop practical strategies that could be adopted by farmers to address the pressing problem on mass mortalities of net-caged and pond reared fishes attributed to persistent and emerging fish parasites. The efficacy of new anti-parasitic agents in consonance with good aquaculture practices will be examined. In addition, the species diversity, morphology and life cycle, and epidemiology of emerging parasites in both marine and freshwater fishes will be investigated. Once pertinent data are generated, prevention and control measures against these parasites could be instituted. |
| **Activity 4**: Epidemiology of the Early Mortality Syndrome (EMS) /Acute Hepatopancreatic Necrotic Disease (AHPND) | Early Mortality Syndrome (EMS) otherwise known as Acute Hepatopancreatic Necrosis Syndrome (AHPNS) is an emerging disease affecting most Southeast Asian Countries whose putative disease-causing agent has been confirmed recently to be Vibrio parahaemolyticus. This study will try to develop protective measures based on the etiological agents together with identification of risk factors and protective factors. Visit to farms with (EMS/AHPNS) outbreaks (Thailand, Vietnam, and Indonesia) will be undertaken to collect samples and other farm data. Samples of EMS/AHPNS –"infected" shrimp will be analyzed using histopathological techniques. Measures to exclude the pathogen |

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| | from the farm, good management practices, good nutrition, and proper handling of the fish to prevent unnecessary stress to the animals will be formulated. From these broad measures, specific protocols to prevent the outbreak of this disease will be developed in cooperation with farmers and hatchery operators. Expected output of the study will be specific recommendations and guidelines to protect shrimp from EMS/AHPNS. (Location: Member Countries concerned) |
| **Activity 5**: Technology extension and demonstration | An important component of an effective aquatic health management system requires trained government and private industry personnel for roles in disease diagnostics, epidemiology, biosecurity, disease emergency management, and scientific research. The availability of trained personnel designated to fill these roles directly impacts the strength of the aquatic animal health management system of a certain country in the region. On-site training/ hands on activities and guided research designed for fish health personnel to keep abreast with diseases confronting important aquatic species in the region is a pragmatic strategy aimed at generating a pool of information essential for the formulation of practical disease prevention and control strategies and enhancement of fish health workers' capacity to plan and execute appropriate and timely scientific research. (Location: Member Countries concerned) |
| **Activity 6**: Publication | Manuals, posters, pamphlets and flyers describing disease prevention methods will be published and distributed. |
| **Activity 7**: Annual progress meeting and international workshop | **Sub-activity 7.1** Annual progress meeting<br>Annual meeting organized by SEAFDEC/AQD is held to review the project achievement. Evaluators will be invited to join the meeting to review/evaluate the project achievements.<br>**Sub-activity 7.2** International workshop<br>The workshop, not only receive participants from member countries, but also invite expert scientists as key note speakers to facilitate to spread and exchange brand-new information on fish health management between SEAFDEC and various institutions. |
| **Activity 8**: Coordination by the project leader | The project leader coordinates and encourages the research, training and dissemination, and also facilitate information exchange not only between activities but also among member countries. |

### 3.3 Activity, Sub-activity and Proposed Budget for 2014-2019 (*in case of 5 year project from 2015*)

**(**Unit: USD**)**

| Activity | Sub-Activity | Y2 2015 | Y3 2016 | Y4 2017 | Y5 2018 | Y6 2019 |
|---|---|---|---|---|---|---|
| **Activity 1**: Development and acceleration of rapid and effective fish and shrimp health management | | 11,000 | 8,800 | 8,800 | 18,800 | 8,800 |

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| **Activity 2**: Enhancement of efficacy of vaccine treatment in tropical cultured species | **Sub-activity 2.1**: Enhancement of vaccine efficacy for the prevention of viral nervous necrosis in high value marine fish | 11,000 | 8,800 | 8,800 | 15,800 | 8,800 |
| | **Sub-activity 2.2**: Application of adjuvants, carriers and RNAi technology to enhance the antiviral immune response of shrimp to WSSV | 11,000 | 8,800 | 8,800 | 10,800 | 10,800 |
| **Activity 3**: Establishment of protective measures against persistent and emerging parasitic diseases of tropical fish | | 11,000 | 8,800 | 8,800 | 15,800 | 8,800 |
| **Activity 4**: Epidemiology of the Early Mortality Syndrome (EMS) /Acute Hepatopancreatic Necrotic Disease (AHPND) | | 11,000 | 8,800 | 8,800 | 8,800 | 8,800 |
| **Activity 5**: Technology extension and demonstration | | 15,000 | 14,000 | 14,000 | 24,000 | 14,000 |
| **Activity 6**: Publication | | 0 | 0 | 0 | 0 | 0 |
| **Activity 7**: Annual progress meeting and international workshop | **Sub-activity 7.1**: Annual progress meeting | 5,000 | 4,000 | 4,000 | 4,000 | 0 |
| | **Sub-activity 7.2** : International workshop | 0 | 0 | 0 | 0 | 0 |
| **Activity 8**: Coordination by the project leader | | 6,438 | 4,159 | 5,000 | 5,000 | 5,510 |
| | Sub-Total Budget | 81,438 | 66,159 | 67,000 | 103,000 | 65,510 |

**PART II: ACHIEVEMENT OF 2019 PROJECT IMPLEMENTATION**

**1. Achievements of the Project Implementation for the present year <2019>:**

**Activity 1)** Preliminary infection experiment for the determination of $LD_{50}$ for the 4 weight ranges has been conducted. The time course experiments were conducted to determine the threshold levels of WSSV infection using 4 weight ranges have been completed. Standard curve has been established using WSSV plasmid. Threshold infection level for WSSV has been determined. The time course experiment on AHPND using the three (3) body weight ranges (ABW= 3-5g, 15-18g, 20g-up) was already conducted. The standard curve for the real-time PCR was established.

**Activity 2.1)** The field efficacy of the inactivated nervous necrosis virus (NNV) vaccine against viral nervous necrosis (VNN) in grouper (*Epinephelus coioides*) was verified as evidenced by the

upregulation of NNV-neutralizing antibodies in the sera of vaccinated fish examined at different time points post-vaccination coupled by the conferment of protection, i.e. higher survival rate, in vaccinated fish challenged with the homologous NNV. On the contrary, NNV-neutralizing antibodies were not detected in surviving and dead unvaccinated fish. Additionally, high NNV titers ($>10^9$ $TDID_{50}$/g) were quantified in the brains of dead unvaccinated fish.

**Activity 2.2)** Effective dose and frequency of oral administration via feeding as well as optimum ratio of dsRNA to rVP28 were determined. Verification of results in a pond trial is ongoing.

**Activity 3)** Efficacy of orally administered garlic extract using allicin powder against Trichodina sp. in Nile tilapia has been tested and the efficacy of garlic extract against infection with sealice in pompano has been conducted.

**Activity 4)** Efficacy of aged seawater against AHPND was evaluated.

**Activity 5)** On-site training courses are conducted in Myanmar and Cambodia in the last quarter of 2019. Annual and semi-annual progress meetings were conducted every year.

**Activity 7)** The International workshop on "Promotion of Sustainable Aquaculture, Aquatic Animal Health and Resource Enhancement in Southeast Asia (SARSEA 2019)" was conducted at Iloilo city, 25-27 June 2019.

**2. Information of Present Year Activity including Involved Stakeholders:**

| List of Actual Sub-activity | Type of activity* | Number of Participants | | | Spent Budget (USD) |
|---|---|---|---|---|---|
| | | MCs | SEAFDEC | Others | |
| **Activity 1** Development and acceleration of rapid and effective fish and shrimp health management | Research | | | | 8,800 |
| **Activity 2** Enhancement of efficacy of vaccine treatment in tropical cultured species | | | | | |
| **Sub-activity 2.1** Enhancement of vaccine efficacy for the prevention of viral nervous necrosis in high value marine fish | | | | | |
| **Sub-activity 2.2**: Application of adjuvants, carriers and RNAi technology to enhance the antiviral immune response of shrimp to WSSV | Research | | 11 (5) | | 4,000 |
| **Activity 3**: Establishment of protective measures against persistent and emerging parasitic diseases of tropical fish | Research | | | | |
| **Activity 4**: Epidemiology of the Early Mortality Syndrome (EMS) /Acute Hepatopancreatic Necrotic Disease (AHPND) | | | | | |
| **Activity 5** Technology extension | II: Training | | | | 14,000 |

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| and demonstration | Not applicable at the moment. | | | | |
| **Activity 6**: Publication | | | | | |
| **Activity 7**: Annual progress meeting and international workshop | Workshop | 19 (7) | 33 (14) | 1 (0) | 0 |

***Remarks) Regarding the number of participants, please indicate total number of participants (and number of female participants within), such as 20 (10).***

**3. Achievements and Expected Outcome/Outputs of the Activity:**

| Planned activity | Expected outcome/output | Achievements |
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| **Activity 1** Development and acceleration of rapid and effective fish and shrimp health management | Determination of threshold infection levels for WSSV and other pathogens such as $VP_{AHPND}$ at different age/weight ranges | Standard curve has been established using WSSV plasmid. Load of the viral stock was determined using q-PCR to be $1.6x10^7$ copies/g. The viral load from natural infection was also measured. Viral load for one-step positive tissues ranges from $3.2x10^9$ to $5.1x10^{10}$ copies/g while the range for the nested positive tissues were from $7.4x10^3$ to $1.2x10^5$ copies/g. In the artificial infection (time course experiment), the one-step positive tissues range from 1.2 to 5.1 X $10^9$ copies/g while the range for nested is from 3.3 to 9.3 X $10^6$ copies/g. The threshold level of infection for WSSV was between $10^5$ and $10^7$ copies/g where mortality is not yet observed. The results also show that threshold level of infection for WSSV is not weight dependent. It should be noted however, that clinical signs of WSSV such as white spots where not observed in the artificial infection experiment. The q-PCR protocol was optimized in TF 5. The protocol was successful and can be used for diagnostic purposes<br>For AHPND, the time course experiments for the three (3) body weight ranges (ABW= 3-5g, 15-18g, & 20g-up) were already conducted. The time course experiment for the last body weight range (ABW= 7-8 g) will be conducted in the last quarter of 2019. The refinement of the standard curve for the real-time PCR is still ongoing. The standard curve samples were already established. |
| **Activity 2** Enhancement of efficacy of vaccine treatment in tropical cultured species | | |
| **Sub-activity 2.1** Enhancement of vaccine efficacy for the prevention of viral nervous necrosis in high value marine fish | Field efficacy of formalin-inactivated NNV vaccine elucidated in pond-reared | Grouper juveniles (MBW: 8.3±1.2 g) intraperitoneally injected with 100 µl of inactivated NNV vaccine (pre-inactivation titer: $10^{9.2}$ $TCID_{50}$/ml) exhibited neutralizing antibody titers from Day 30 (mean titer 1:1792±701) to Day 150 (1:704±351) with the highest titer observed at |

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| | groupers | Day 60 (1:6656±3435) post-vaccination. Because no mortality was encountered in both vaccinated and unvaccinated fish during the course of the pond experiment, Day 30 post-vaccinated (n=20; MBW: 21±3.4 g) and L15-injected/control (n=20; 20.6±1 g) fish were intramuscularly challenged with NNV ($10^{6.5}$ $TCID_{50}$/fish). Nil and 25% mortality were respectively obtained in both vaccinated and unvaccinated fish. NNV titers in the brains and kidneys of dead unvaccinated fish ranged from $10^{10.9}$~$10^{11.4}$ $TCID_{50}$/g and $10^{8}$~$10^{8.9}$ $TCID_{50}$/g, respectively. On the contrary, NNV was not detected in the brains and kidneys of any vaccinated fish examined. Additionally, NNV-challenge of Day 120 vaccinated (n=20; 178±27 g) and L15-injected/control (n=20; 176±19 g) fish likewise resulted in nil mortality, suggesting an age or weight dependent susceptibility to NNV.<br><br>Recently, IM injection of sublethal dose of NNV in pompano juveniles (MBW: 4.7±1.7 g) resulted in 0~15% mortality rate. When these surviving fish were re-challenged with a lethal dose of NNV at 1 and 2 months post-primary sublethal NNV injection, none of these fish died nor manifested any VNN associated symptoms/ signs. On the contrary, control (naïve) fish groups resulted in 70~80% mortality rates. Determination of antibody and NNV titers in the sera of fish (primary and post-NNV challenge) collected at different point post-NNV injection and brains of dead fish, respectively, is ongoing. Current findings indicate that in natural NNV infection, upregulation and subsequent proliferation of anti-NNV neutralizing antibodies play an important role in suppressing or controlling the progression of the disease. |
| **Sub-activity 2.2**<br>Application of adjuvants, carriers and RNAi technology to enhance the antiviral immune response of shrimp to WSSV | To develop a vaccination scheme using a combination of the two antiviral treatments (rVP28 vaccination and rVP28 RNAi treatment).<br><br>To develop a low-cost delivery protocol for the antiviral treatments in tanks. | dsRNA was produced using a low-cost bacterially expressed dsRNA production method. The efficacy of dsRNA was tested in several challenge experiments using various dsRNA doses, different frequency of dsRNA administration, and inclusion of heterologous dsRNA to test the specificity of gene silencing. The best treatment was determined to be a dose of 20 µg/shrimp administered 4 times over 28 days (total = 80 µg/shrimp). Furthermore, the silencing was found to be specific to VP28 dsRNA. Moreover, production of rVP28 and dsRNA and determination of their encapsulation efficiency and yield in chitosan and alginate microparticles were conducted. In 2019, optimum ratio of dsRNA to rVP28 in microparticle carriers was determined to be 1:3. Pond trial to verify the above results is ongoing. |

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| **Activity 3** Establishment of protective measures against persistent and emerging parasitic diseases of tropical fish | 1) To test the efficacy orally administered garlic (*Allium sativum*) extract (allicin powder) against *Trichodina* sp. in Nile tilapia (*Oreochromis niloticus).*<br>2) To assess the ability of efficacy of garlic extract against infection with sea lice (*Caligus sp*.) in pompano (*Trachinotus blochii*) . | 1) Results showed that tilapia fed with allicin powder-supplemented diets showed significant reduction in the prevalence and mean intensity of ciliate parasites (*Trichodina* sp.) as compared to the control. Histopatholgical changes such as fragmented muscle and disarranged collagen bundle along the muscle of tilapia fed positive control were seen. Gills of fish fed with control diet showed hyperplasia and fusion of gill lamellae. Liver of tilapia fed with Diet 5 (5g/kg) showed congestion and vacoulation of the hepatocytes. In vitro results of garlic extract (allicin powder) efficacy testing on Trichodina revealed that the time required for killing of *Trichodina* parasites at lowest concentration (150 ppm) was 45 mins. However, at the highest concentration (400 ppm) it required 10 mins to kill all parasites. Bath treatment will be conducted upon the availability of infested tilapia with Trichodinids.<br>2) On the other hand, toxicity trials were performed to determine the concentration of garlic extract as therapeutic immersion treatment for sea lice in pompano (*Trachinotus blochii*)**.** Results yielded from the experiment showed that the median lethal concentration (LC50) of allicin powder to pompano for 24, 48, 72 and 96 h of exposure were 29.18, 23.31, 16.79 and 6.64 mg/L respectively. Histopathology revealed that the gills of *T. blochii* in the control group showed no signs of abnormalities while the gills of test fish with the supplemented garlic extract diet showed epithelial lifting, hyperplasia, and fusion of the secondary lamellae. While the liver of test fish in the control group showed vacoulation of the hepatocytes, the liver of fish fed with supplemented garlic extract diet, on the other hand, showed severe congestion of blood vessels and vacoulation, pyknosis and dilations of sinusoids. *In-vitro* parasite survival experiment, oral treatment and bath treatment will be undertaken. |
| **Activity 4** Epidemiology of the Early Mortality Syndrome (EMS)/ Acute Hepatopancreatic Necrotic Disease (AHPND) | To clarify the efficiency of green-water system using brown mussel against AHPND in a simulated tank experiment.<br><br>To investigate other alternative measures that will mitigate the effect | The efficiency of raw seawater aged at different periods (28 days, 14 days, 7 days and 0 day) in shrimp with high (one-step PCR positive) and low (nested PCR-positive) $VP_{AHPND}$ infection were investigated. Significantly higher shrimp survival was observed in shrimp with low $VP_{AHPND}$ infection compared to shrimp with high $VP_{AHPND}$ infection. Among shrimp with low $VP_{AHPND}$ infection, higher survival was observed in those maintained in seawater aged for 14-28 days (70% and 63%, respectively) compared to those maintained in seawater aged for 7 days (48%) and non-aged seawater (39%). Survival in shrimp with |

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| | of AHPND aside from the green-water system | high infection maintained in 0 to 4 days ranged from 8.4 to 19%.<br>Experiment to clarify the effect of brown mussel was not done due to the lack of brown mussel source. |
| **Activity 5** Technology extension and demonstration | On-site training courses on basic fish bacteriology, antimicrobial assay and disinfection techniques implemented in Cambodia and Myanmar | Coordination with the DOF of Myanmar to conduct an on-site training that specifically focuses on issues pertinent bacterial aeromonad septicemia in cultured freshwater fish species is currently being undertaken. The target date of training will be in November 2019. |
| **Activity 6**: Publication | To print proceedings for workshop | The proceedings of International workshop on "Promotion of Sustainable Aquaculture, Aquatic Animal Health and Resource Enhancement in Southeast Asia (SARSEA 2019)" to be printed in first quarter of 2020. |
| **Activity 7**: Annual progress meeting and international workshop | To hold international workshop | The International workshop on "Promotion of Sustainable Aquaculture, Aquatic Animal Health and Resource Enhancement in Southeast Asia (SARSEA 2019)" was conducted at Iloilo city from 25-27 June, 2019. There were fifty-three participants. Nineteen participants from member countries, one participant from other country and thirty-three participants from SEAFDEC were attended. The participants also reported on the status of sustainable aquaculture, resource enhancement and aquatic animal health of their respective countries. The participants could be updated on the issues related to sustainable aquaculture, aquatic animal health and resource enhancement, and will put forward recommendations to address the issues. |

**4. List of Completed Publications and Others (*e.g.* technical report, VDO, presentation file, etc.):**

| List of completed publications for the year 2019 | Type of media | Attached e-file |
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| **1. Pakingking Jr.,R**, Bautista, NB and de Jesus-Ayson, EG (2018) Characterization of *Vibrio* isolates recovered from the eyes of cage-cultured pompano (*Trachinotus blochii*) infested with caligid parasites (*Lepeophtheirus spinifer*). Bulletin of the European Association of Fish Pathologists 38 (1): 35-41 | Journal publication | |
| **2 Pakingking Jr., Rolando**, de Jesus-Ayson, Evelyn Grace, Reyes, Ofelia, Bautista, Norwell Brian (2018) Immunization regimen in Asian sea bass (*Lates calcarifer*) broodfish: a practical strategy to control vertical transmission of nervous necrosis virus during seed production. Vaccine 36: 5002–5009 | Journal publication | |

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| **3. R Pakingking Jr. R. (2018)** Immunization regimen in high value marine broodfish: a pragmatic strategy to control vertical transmission of nervous necrosis virus during seed production (Oral Presentation) Asian Aquaculture 2018: Celebrating Asian Aquaculture, Asian Institute of Technology Convention Center, Klong Luang, Pathumthani, Thailand, 3-6 December 2018 | Book of Abstract | |
| **4. Leobert D. de la Peña** (2019) Establishment of Threshold Infection Levels for WSSV and Other Pathogens such as $VP_{AHPND}$ in Penaeid Shrimp. Presented at the International workshop on "Promotion of Sustainable Aquaculture, Aquatic Animal Health and Resource Enhancement in Southeast Asia (SARSEA 2019)", 25-27 Jun 2019, Iloilo Philippines. | Book of Abstract | |
| **5. Rolando Pakingking Jr.** and Evelyn Grace de Jesus-Ayson (2019) Enhancement of Vaccine Efficacy for the Prevention of Viral Nervous Necrosis in High Value Marine Fish. Presented at the International workshop on "Promotion of Sustainable Aquaculture, Aquatic Animal Health and Resource Enhancement in Southeast Asia (SARSEA 2019)", 25-27 Jun 2019, Iloilo Philippines. | Book of Abstract | |
| **6. Edgar C. Amar** (2019) Application of Adjuvants, Carriers and RNAi to Enhance the Antiviral Immune Response of Shrimp to WSSV. Presented at the International workshop on "Promotion of Sustainable Aquaculture, Aquatic Animal Health and Resource Enhancement in Southeast Asia (SARSEA 2019)", 25-27 Jun 2019, Iloilo Philippines. | Book of Abstract | |
| **7. Gregoria Erazo-Pagador** (2019) Establishment of Protective Measures Against Persistent andEmerging Parasitic Diseases of Tropical Fish. Presented at the International workshop on "Promotion of Sustainable Aquaculture, Aquatic Animal Health and Resource Enhancement in Southeast Asia (SARSEA 2019)", 25-27 Jun 2019, Iloilo Philippines. | Book of Abstract | |
| **8. Eleonor A. Tendencia** (2019) Epidemiology of the Early Mortality Syndrome (EMS). Presented at the International workshop on "Promotion of Sustainable Aquaculture, Aquatic Animal Health and Resource Enhancement in Southeast Asia (S ARSEA 2019)", 25-27 Jun 2019, Iloilo Philippines. | Book of Abstract | |

**5. Evaluation from Participants of Member Countries for WS and Training Course (if available):**

| Planned activity | Evaluation/ Views from Participants |
|---|---|
| **Activity 1** Development and acceleration of rapid and effective fish and shrimp health management | Not applicable |
| **Activity 2** Enhancement of efficacy of vaccine treatment in tropical cultured species | |

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| **Sub-activity 2.1** Enhancement of vaccine efficacy for the prevention of viral nervous necrosis in high value marine fish | Not applicable |
| **Sub-activity 2.2**: Application of adjuvants, carriers and RNAi technology to enhance the antiviral immune response of shrimp to WSSV | Not applicable |
| **Activity 3**: Establishment of protective measures against persistent and emerging parasitic diseases of tropical fish | Not applicable |
| **Activity 4**: Epidemiology of the Early Mortality Syndrome (EMS) /Acute Hepatopancreatic Necrotic Disease (AHPND) | Not applicable |
| **Activity 5** Technology extension and demonstration | Not applicable at the moment. To be conducted Dec. 2016 |
| **Activity 6**: Publication | |
| **Activity 7**: Annual progress meeting and international workshop | The International workshop on "Promotion of Sustainable Aquaculture, Aquatic Animal Health and Resource Enhancement in Southeast Asia (SARSEA 2019)" was highly evaluate from Participants (average mark was 4.6 out of 5 ). |

**6. Major impacts/issues:**

**Activity 2.1)** Unprecedented outbreaks of VNN in pond-reared groupers, particularly at the juvenile stages, could be avoided since single administration of the monovalent inactivated NNV vaccine effectively upregulated the production of NNV-neutralizing antibodies and concomitant conferment of protection against VNN. Moreover, the use of the inactivated vaccine in other warm-water marine fish species such as sea bass and pompano is highly feasible since VNN-induced mortalities in these fish species have been found to be caused by NNV strains belonging to a single genotype, i.e. RGNNV type.

## PART III: ACHIEVEMENTS IN OVERALL PROJECT DURATION

**1. Abstract of Achievements in the Overall Project Duration *(project duration 6 years (5years))*:**

**Activity 1)** Determination of threshold infection levels for WSSV in shrimp at different ages/weight ranges were already established. The established threshold level will enable the farmers to strictly monitor the health status so that early and effective intervention strategies can be implemented and will serve as a reference in the monitoring and diagnostic schemes in the farm level, if it is still safe or dangerous. The study already completed the establishment of threshold infection level for the WSSV while for $VP_{AHPND,}$ a few more activities are needed to finish the study at the end of 2019.

**Activity 2.1)** Single administration of the monovalent formalin-inactivated NNV vaccine in pompano and groupers was proven effective in upregulating the production of NNV-neutralizing antibodies and concomitant conferment of protection against VNN. Moreover, the potential use of this inactivated vaccine against NNV infection in other susceptible warm-water marine fish species particularly during the early phase of grow-out culture in earthen ponds or floating net-cages in the open sea is very much feasible since mortalities of these fish species have been found to be caused by NNV strains belonging to a single genotype, i.e. RGNNV type. Additionally, current trial, i.e. IP injection of sublethal dose of NNV in pompano juveniles followed by subsequent injection with a lethal dose of NNV at 1 and 2 months post-primary sublethal NNV injection, resulted in nil mortality coupled by the absence of any VNN associated symptoms/ signs; contrary to the control (naïve) fish which resulted in 70~80% mortality rates. Although determination of antibody and NNV titers in the sera of fish (primary and post-NNV injection) collected at different time points post-NNV injection and brains of dead fish, respectively, is still ongoing, current findings clearly indicate that in fish naturally infected with NNV, the humoral immune response plays an important or perhaps major role in suppressing or controlling the proliferation of NNV in the nervous tissues of infected fish, thereby abrogating the progression of the disease. The potentiality of using this approach as a practical vaccination strategy against NNV infection in susceptible fish species warrants further investigation.

**Activity 2.2)** dsRNA was produced using a low-cost bacterially expressed dsRNA production method. The optimum dose was determined to be 20 µg/shrimp administered 4 times over 28 days (total = 80 µg/shrimp). Furthermore, the silencing was found to be specific to VP28 dsRNA. The encapsulation efficiency and yield in chitosan and alginate microparticles were found to be adequate. The dsRNA to rVP28 ratio in microparticle carriers was determined to be 1:3. Pond trial to verify the above results is ongoing.

**Activity 3)** Experimental transmission of gill monogenean *Pseudorhabdosynochus lantauensis o*n cultured orange spotted grouper (*Epinephelus coiodes*) fingerlings was conducted. The study also examined the anti-parasitic effect of garlic (*Allium sativum*) powder extract (allicin) against gill monogenean parasites (*P. lantauensis*), ciliates (*Trichodina* sp.) and sea lice (*Lepeoptheirus spinifer*, *Caligus* sp.) infecting grouper (*E. coiodes*), Nile tilapia (*Oreochromis niloticus*) and pompano (*Trachinotus blochii*) respectively. The efficacy of garlic extract was tested *in vitro* and *in vivo*. In addition, treatment of infected fish with garlic-supplemented diets was carried out. Histopathology of gills, liver, and kidney from the *in vitro* and oral treatments were analyzed. The hematocrit, hemoglobin, RBC count, WBC count, and WBC differential count of infected and uninfected fish: *E. coioides*, *O. niloticus* and *T. blochii* were also undertaken.

**Activity 4)** One of the most recent diseases affecting the shrimp industry is the early mortality syndrome (EMS). EMS characterized by observed mortality in shrimp within the first 35 days of culture is due to several diseases, one of which is the acute hepatopancreatic disease (AHPND). Re Immersion in $10^7$ cfu/ml $VP_{AHPND}$, either in the culture water or in a bacterial suspension for 15 minutes, can cause mortality in healthy shrimp. Tank experiments done suggested that exposure to $35^oC$, 28ppt and 10 ppt increase the risk of shrimp mortality due to $VP_{AHPND}$. On the other hand, use of green water that has been stocked with siganid for not less than 2 weeks might provide some protection against the disease. Use of siganid water to culture shrimp improves shrimp growth and survival. Use of mussel may also improve shrimp survival but needs further investigation; whereas, the use of macro algae is not effective against $VP_{AHPND}$. Use of seawater aged for not less than 14 days may improve survival in shrimp with low $VP_{AHPND}$ infection but in those with high $VP_{AHPND}$ infection.

**Activity 5)** The on-site training courses conducted in Myanmar, Cambodia and Lao PDR primarily delved on health management of parasitic and bacterial diseases of cultured freshwater fish species. Because of the series of on-site trainings conducted, fish health staff (FHS) have been capacitated with the necessary knowledge and skills needed for the accurate laboratory diagnosis of bacterial and parasitic diseases affecting cultured freshwater fish species. Lectures focusing on major bacterial and parasitic diseases affecting cultured freshwater fishes in Myanmar, Cambodia and Lao PDR enabled fish health personnel to keep abreast with the latest issues on persistent and emerging transboundary diseases of aquatic organisms in the region. The theoretical knowledge acquired by the trainees have been further enhanced by the skills they learned from the hands-on exercises such as necropsy, parasite identification, bacteriological techniques including asepsis, biochemical characterization tests, in vitro drug sensitivity test, and infection bioassay among others. Notably, several staff from the DOF, academe, and other government and private agencies were able to avail of the on-site training courses by far conducted.

**Activity 7)** Annual and semi-annual progress meeting were conducted. The International workshop on "Promotion of Sustainable Aquaculture, Aquatic Animal Health and Resource Enhancement in Southeast Asia (SARSEA 2019)" was conducted in 2019.

## 2. Implemented Activities/sub-activities in the Overall Project Duration:

| List of Activities | Description of Implemented Activities |
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| **Activity 1** Development and acceleration of rapid and effective fish and shrimp health management | Earlier, optimization of the protocols for conventional and real-time PCR was conducted. The optimized protocols were used in the determination of the threshold levels for WSSV and AHPND. Preliminary infection experiment for the determination of $LD_{50}$ for the 4 weight ranges has been conducted. The time course experiments were conducted to determine the threshold levels of WSSV infection using 4 weight ranges have been completed. Standard curve has been established using WSSV plasmid. Threshold infection level for WSSV has been determined. The time course experiment on AHPND using the three (3) body weight ranges (ABW= 3-5g, 15-18g, 20g-up) was already conducted. The standard curve for the real-time PCR was established. These optimized protocols can be used in the detection of the target pathogens under the Diagnostic Services of the Fish Health Section. |
| **Activity 2** Enhancement of efficacy of vaccine treatment in tropical cultured species | |
| **Sub-activity 2.1** Enhancement of vaccine efficacy for the prevention of viral nervous necrosis in high value marine fish | The field efficacy of the previously developed inactivated Philippine strain of NNV vaccine was pilot tested in pompano reared in floating net cages in the open sea and in groupers reared in earthen ponds. The efficacy of the inactivated NNV vaccine, i.e. its immunogenicity and ability to confer protection against NNV infection, was clearly elucidated in these fish species, particularly in groupers, as evidenced by the presence of neutralizing antibodies in the sera of vaccinated fish examined at different time points post-vaccination. Moreover, when groupers were experimentally challenged with the homologous virus, higher survival rate was obtained in vaccinated fish compared with the control fish coupled by the absence and very high ($\geq 10^9$ $TCID_{50}$/ g) NNV titers in the brains of vaccinated fish and unvaccinated fish, respectively. Recently, the potentiality of using sublethal dose of NNV as |

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| | practical vaccination strategy in pompano juveniles was conducted. IM injection of sublethal dose of NNV in pompano juveniles resulted in 0~15% mortality rate. None of these surviving fish re-challenged with a lethal dose of NNV at 1 and 2 months post-primary sublethal NNV injection died nor manifested any VNN associated symptoms/ signs, contrary to 70~80% mortality rates obtained for control (naïve) fish groups. Determination of antibody and NNV titers in the sera of fish (primary and post-NNV challenge) collected at different point post-NNV injection and brains of dead fish, respectively, is ongoing. |
| **Sub-activity 2.2**: Application of adjuvants, carriers and RNAi technology to enhance the antiviral immune response of shrimp to WSSV | The efficacy of dsRNA treatments in protecting shrimp against WSSV<br>was evaluated. Then, a scheme combining the two antiviral treatments (rVP28 vaccination and rVP28 RNAi) was developed, including a low-cost delivery protocol for the antiviral treatments in tanks. A field trial using orally (feed)- delivered antiviral treatments is ongoing. |
| **Activity 3**: Establishment of protective measures against persistent and emerging parasitic diseases of tropical fish | Experimental transmission of *P. lantauensis* in *E. coioides* juveniles showed 100, 80.0, 53.3% mortality in 10 fish/20L challenged with 800, 400 and 200 oncomiracidia, respectively, at day 4 post-challenge. No mortality was observed in fish infected with 100 oncomiracidia and the control (0 oncomiracidium). Histopathological analysis showed extensive hyperplasia and hypertrophy in the gill filaments of infected grouper challenged with 800 oncomiracidia. Hematological analysis revealed that hematocrit, hemoglobin and red blood cell were lower in infected fish than healthy individuals. In contrast, number of white blood cells was higher in infected fish compared to non-infected fish. The anti-parasitic effect of garlic (*Allium sativum*) extract (allicin powder) against monogenean parasites (*P. lantauensis*) ahowed that the 96 h ($LC_{50}$) of garlic extract for 24, 48, 72 and 96 h of exposure are 6.24, 5.94,.5.15 and 3.659 ml/L respectively. *In vitro* trials revealed that at 2.5 ppm garlic extract, parasites detached and died at 20 and 40 min respectively, whereas, at 30 ppm, detachment and death occurred at 5 and 10 min respectively. Oral treatment of garlic (*A. sativum*) against *P. lantauensis* showed that fish fed with diets supplemented (0.10%, 0.50% and 1.0% allicin powder) showed reduced prevalence and mean intensity of parasites as compared to the control.. Effect of garlic extract (allicin powder) as antiprotozoal to control trichodinosis in tilapia (*Oreochromis niloticus*) showed the LC50 values of garlic extract for 24, 48, 72 and 96 hours were 398.1, 360.7, 316.21 and 208.95 ppm respectively. Oral treatments using allicin powder-supplemented diet showed that tilapia fed with allicin showed reduced prevalence of *Tilapia sp.* infection as compared to the control i.e. 1.25. 2.5, 3.75 and 5 g/kg were 72%, 65%, 56%, 37% respectively, as compared to 100% in the control. Mean intensity of *Tilapia sp.* was reduced in fish fed with allicin diets as compared to the control. Histopathological analysis was conducted. Results showed that hematocrit, hemoglobin and red blood cell count were lower in infected fish than in healthy individuals. White blood cell count was higher in infected fish compared to non-infected fish. *In vitro* results of garlic extract (allicin powder) on Trichodina revealed that the time required for killing of *Trichodina* parasites at lowest concentration (150 ppm) was 45 mins. and at 400 ppm was 10 mins. Efficacy of garlic (*A.* |

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| | *sativum*) extract as ant-parasitic against sea lice on pompano (*Trachinotus blochii*) showed that (LC50) values for 24, 48, 72 and 96 h of exposure were 29.18, 23.31, 16.79 and 6.64 mg/L respectively. Analysis of histopathology was conducted. *In-vitro* parasite survival experiment, oral treatment and bath treatment will be undertaken. |
| **Activity 4** Epidemiology of the Early Mortality Syndrome (EMS)/ Acute Hepatopancreatic Necrotic Disease (AHPND) | Tanks experiments were done to determine infection method to be used in the investigation of $VP_{AHPND}$ risk factors and possible control measures. Based on farm reports, the role of different temperatures and salinities in shrimp mortalities due to $VP_{AHPND}$ were investigated. Possible protective measures investigated were green water using siganid, macro algae and brown mussels; and the use of aged seawater. |
| **Activity 5** Technology extension and demonstration | The first on-site training course focusing on health management of parasitic diseases of freshwater fish species was conducted at the Aquatic Animal Health Disease Controlling Section, DOF, Tharketa, Yangon, Myanmar last 18 to 22 January 2016. The theoretical and practical aspects of the training mainly delved on major parasitic diseases affecting cultured freshwater fishes in Myanmar. Because bacterial infections persistently affect freshwater fishes cultured in earthen ponds and reservoir, DOF's request to conduct a follow up on-site training course focusing on basic bacteriological techniques was realized from 7 to 11 November 2016. Lectures covered major bacterial diseases of freshwater fishes while hands-on exercises including bacterial isolation, purification, characterization, and infection bioassay were carried out.<br><br>The third on-site training course focusing on health management of parasitic diseases was conducted from 6 to 10 December 2016 at the Fish Health Laboratory, Marine Research and Development Center (MARDeC), Sihanoukville, Cambodia, as per request of its country representative during the SEAFDEC council meeting. Lectures and hands on exercises respectively focused on major bacterial and parasitic diseases and parasite detection in freshwater fishes.<br><br>Additionally, as per Lao PDR's request, a similar on-site training course focusing on health management of parasitic diseases was conducted from 20 to 24 November 2017 at Namxoung Aquaculture and Development Center (NADC), Lao PDR. Lectures delved on major bacterial and parasitic diseases of cultured freshwater fishes while hands-on exercises including fish necropsy, quantitative determination of parasite load, and identification. Updates on tilapia lake virus infection, an emerging and pressing problem besetting the tilapia aquaculture industry in Asia was also presented. Notably, the said training was featured in a national TV news program and national newspaper. |
| **Activity 6**: Publication | The proceedings of International workshop on "Promotion of Sustainable Aquaculture, Aquatic Animal Health and Resource Enhancement in Southeast Asia (SARSEA 2019)" to be printed in first quarter of 2020. |
| **Activity 7**: Annual progress meeting and international workshop | Annual and semi-annual progress meeting were conducted every year. The International workshop on "Promotion of Sustainable Aquaculture, Aquatic Animal Health and Resource Enhancement in Southeast Asia (SARSEA 2019)" was conducted at Iloilo city from |

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| | 25-27 June, 2019. |

**3. Achievements and Outcomes/Outputs of Activities in the Overall Project Duration:**

| List of Activities | Achievements and Outcomes/Outputs of Activities |
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| **Activity 1** Development and acceleration of rapid and effective fish and shrimp health management | The determination of threshold infection levels for WSSV in shrimp at different ages/weight ranges will enable the farmers to strictly monitor the health status so that early and effective intervention strategies can be implemented and will serve as a reference in the monitoring and diagnostic schemes in the farm level, if it is still safe or dangerous. |
| **Activity 2** Enhancement of efficacy of vaccine treatment in tropical cultured species | |
| **Sub-activity 2.1** Enhancement of vaccine efficacy for the prevention of viral nervous necrosis in high value marine fish | Pompano juveniles (MBW: 5.7±0.4 g) were intraperitoneally (IP) vaccinated with inactivated NNV (pre-inactivation titer of 109.2 TCID50/ ml) and reared in floating net cages in the open sea. Fish injected with L-15 medium served as control. Vaccinated fish exhibited neutralizing antibodies as early as Day 30 (1:1493±498), peaked at Day 60 (4267±1260), but thereafter gradually declined at Day 195 (453±203) and Day 210 (293±178) (harvest). On the contrary, all control fish examined did not possess NNV neutralizing antibodies (<1:40). However, protective effect of vaccine-induced antibodies could not be demonstrated since natural NNV infection was not encountered during the course of the experiment. Thus, the field efficacy of the vaccine was subsequently tested in groupers, a highly susceptible species to VNN.<br><br>Grouper juveniles (MBW: 8.3±1.2 g) intraperitoneally injected with 100 µl of inactivated NNV vaccine (pre-inactivation titer: 109.2 TCID50/ml) exhibited neutralizing antibody titers from Day 30 (mean titer 1:1792±701) to Day 150 (1:704±351) with the highest titer observed at Day 60 (1:6656±3435) post-vaccination. Because no mortality was encountered in both vaccinated and unvaccinated fish during the course of the pond experiment, Day 30 post-vaccinated (n=20; MBW: 21±3.4 g) and L15-injected/control (n=20; 20.6±1 g) fish were intramuscularly challenged with NNV (106.5 TCID50/fish). Nil and 25% mortality were respectively obtained in both vaccinated and unvaccinated fish. NNV titers in the brains and kidneys of dead unvaccinated fish ranged from 1010.9~1011.4 TCID50/g and 108~108.9 TCID50/g, respectively. On the contrary, NNV was not detected in the brains and kidneys of any vaccinated fish examined. Additionally, NNV-challenge of Day 120 vaccinated (n=20; 178±27 g) and L15-injected/control (n=20; 176±19 g) fish likewise resulted in nil mortality, suggesting an age or weight dependent susceptibility to NNV.<br><br>Recently, IM injection of sublethal dose of NNV in pompano juveniles (MBW: 4.7±1.7 g) resulted in 0~15% mortality rate. When these surviving fish were re-challenged with a lethal dose of NNV at 1 and 2 months post-primary sublethal NNV injection, none of these fish died nor manifested any VNN associated symptoms/ signs. On the contrary, control (naïve) fish groups resulted in 70~80% mortality rates. Determination of antibody and NNV titers in the sera of fish (primary and post-NNV challenge) collected at different points post-NNV injection and brains of dead fish, respectively, is ongoing. Current findings indicate that |

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| | in natural NNV infection, upregulation and subsequent proliferation of anti-NNV neutralizing antibodies play an important role in suppressing or controlling the progression of the disease. |
| Sub-activity 2.2 Application of adjuvants, carriers and RNAi technology to enhance the antiviral immune response of shrimp to WSSV | dsRNA was successfully expressed and produced using E. coli cells. Several dsRNA doses (0.2, 10, 20, 80, and 120 µg/shrimp) and administration frequencies (1x, daily for 28 d; 4x, and 8x) were tested for protective efficacy against WSSV by challenge experiments. Based on the results of 3 trials, the best treatment was a dose of 20 µg/shrimp administered over 28 days 2 times before and 2 times after challenge (total=80 µg/shrimp). The silencing was specific to VP28 dsRNA. Further tank trials determined that oral delivery using microparticles to encapsulate dsRNA and rVP28 at a 1:3 ratio was the most effective in reducing mortalities upon WSSV infection. A DBS pond trial is being undertaken to confirm results of tank studies and to demonstrate the practicability of the technique under farm conditions. |
| **Activity 3**: Establishment of protective measures against persistent and emerging parasitic diseases of tropical fish | Factors associated with the susceptibility or resistance of fish to some parasitic infection was elucidated. Disease transmission cycle in fish established. Hematological profile of parasites-infected and uninfected fish determined. Practical strategies for the prevention of parasite infestation in fish formulated. |
| **Activity 4** Epidemiology of the Early Mortality Syndrome (EMS)/ Acute Hepatopancreatic Necrotic Disease (AHPND) | Based on the results of the 2 infection methods investigated, immersion in $10^7$ cfu/ml $VP_{AHPND}$, either in the culture water or in a bacterial suspension for 15 minutes, can cause mortality in healthy shrimp. Thus, for the challenge experiments, shrimp was immersed in $10^7$ cfu/ml $VP_{AHPND}$ bacterial suspension for 15 min; this needed smaller volume of bacterial suspension compared to adding bacterial suspension to culture water to a final concentration of $10^7$ cfu/ml. Results of the tank experiments suggested that 35°C, 28ppt and 10 ppt increase the risk of shrimp mortality due to $VP_{AHPND}$. On the other hand, use of greenwater that has been stocked with siganid for not less than 2 weeks might provide some protection against the disease. Use of siganid water to culture shrimp improves shrimp growth and survival. Use of mussel may also improve shrimp survival but needs further investigation; whereas, the use of macro algae is not effective against $VP_{AHPND}$. Use of seawater aged for not less than 14 days may improve survival in shrimp with low $VP_{AHPND}$ infection but in those with high $VP_{AHPND}$ infection. |
| Activity 5 Technology extension and demonstration | Four on-site training courses have by far been conducted, i.e. 2 in Myanmar (1st and last quarter of 2016), 1 in Cambodia (last quarter of 2016), and 1 in Lao PDR (last quarter of 2017). The themes of the trainings already conducted in these aforementioned countries were in conjunction with the requests, i.e. based on identified needs or problems on persistent or emerging fish diseases, of the concerned SEAFDEC member country representatives. The follow up on-site training on basic bacteriological techniques supposedly scheduled for 2018 in Cambodia was not implemented due to the delayed approval of the proposal. To date, a total of 57 individuals have attended the on-site training conducted in Myanmar (n=31), Cambodia (11), and Lao PDR (15).<br><br>An on-site training course on Health management of bacterial and parasitic diseases of freshwater fish species was successfully carried out at the Aquatic Animal Health Disease Controlling Section, DOF, Tharketa, Yangon, Myanmar from 18 to 22 January 2016. Eleven FH |

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| | officers participated in the training. The theoretical and practical aspects of the training primarily focused on major parasitic diseases affecting cultured freshwater fishes in Myanmar. Because bacterial infections have been recognized as a serious problem among freshwater fish species cultured in earthen ponds and reservoir in Myanmar, a follow up on-site training delving on Basic Bacteriological Techniques was implemented from 7 to 11 November 2016. A total of 20 participants, i.e. 15 and 5 from the DOF and academe (Yangon University), respectively, attended the training and proposal writing workshop. Lectures covered major bacterial diseases of freshwater fishes while hands-on exercises included bacterial isolation, purification, characterization, and infection bioassay.<br><br>As per request of Cambodia's country representative, an on-site training focusing on Parasitic Diseases of Freshwater Fishes was conducted from 6 to 10 December 2016 at the Fish Health Laboratory of the Marine Research and Development Center (MARDeC), Sihanoukville, Cambodia. Staff of the Department of Aquaculture Development (n=5), National Aquaculture Research and Development Institute (2), Freshwater Aquaculture and Development Center (2), and MARDeC (2) participated in the training.<br><br>Additionally, as per Lao PDR's request, a similar on-site training course focusing on Parasitic Diseases of Freshwater Fishes was conducted from 20 to 24 November 2017 at Namxoung Aquaculture and Development Center (NADC), Namxoung, Lao PDR. A total of 15 participants, i.e. NADC (n=3); National Animal Health Laboratory (1); Dongkhamxang Agriculture Technical School (2); Living Aquatic Resources Research Center (2); Lao - Singapore Demonstration Fish Hatchery (2); Vientiane Province Technical College (VPTC) (2); National University of Lao PDR (1); Army Agriculture Center (2); and students from VPTC (3) attended the training. Lectures highlighted the major bacterial and parasitic diseases currently affecting cultured freshwater fishes in SE Asia. Hands-on exercises delved on fish necropsy, quantitative determination of parasite load in the gills and skin of fish and parasite identification. Additionally, updates on tilapia lake virus infection, an emerging and pressing problem currently besetting the tilapia aquaculture industry in Asia was presented. Notably, the said training was featured in a national TV news program and national newspaper. |
| **Activity 6**: Publication | |
| **Activity 7**: Annual progress meeting and international workshop | Annual and semi-annual progress meeting were conducted every year, it contributed for proper practice of the project. The International workshop on "Promotion of Sustainable Aquaculture, Aquatic Animal Health and Resource Enhancement in Southeast Asia (SARSEA 2019)" was conducted at Iloilo city from 25-27 June, 2019. There were fifty-three participants. Nineteen participants from member countries, one participant from other country and thirty-three participants from SEAFDEC were attended. The participants also reported on the status of sustainable aquaculture and resource enhancement and aquatic animal health of their respective countries. The participants could be updated on the issues related to sustainable aquaculture, aquatic animal health and resource enhancement, and will put forward recommendations to address the issues. |

**4. Evaluation and Major Impacts/Issues in the Overall Project Duration:**

**Activity 2.2)** Low cost delivery and enhanced efficacy are needed in enhancing the resistance of shrimp by the application of immune stimulating or virus-inhibiting compounds in shrimp aquaculture. These interventions are often shown to be efficacious in *in vitro* and tank trials but field trials are mostly non-existent. Adoption by farmers can be facilitated when these interventions are verified under field conditions. Field trials are expensive to conduct. The project plans to recycle harvest income to pay for some inputs like feeds and life support systems.

**Activity 3)** The results of this study demonstrated the potential of garlic as a natural alternative to the current use of chemical treatments for parasitic infestations in tropical fish.

**Activity 7)** The International workshop on "Promotion of Sustainable Aquaculture, Aquatic Animal Health and Resource Enhancement in Southeast Asia (SARSEA 2019)" was highly evaluate from Participants (average mark was 4.6 out of 5 ). The participants could be updated on the issues related to sustainable aquaculture, aquatic animal health and resource enhancement, and will put forward recommendations to address the issues.

**5. Publications and Others (*e.g.* technical report, VDO, presentation file, etc.):**


Maria Rowena R. Romana-Eguia, Fe D. Parado-Estepa, Nerissa D. Salayo and Ma, Junemie Hazel Lebata-Ramos edit. (2015) Resource Enhancement and Sustainable Aquaculture Practices in Southeast Asia (RESA2014). Proceedings of the international Workshop, ISBN: 978-971-9931-04-1, SEAFDEC AQD

Pakingking Jr. R. Aquatic Animal Health Activities of the Fish Health Section, SEAFDEC/AQD. NACA 2015. Fourteenth Meeting of the Asia Regional Advisory Group on Aquatic Animal Health: Report of the Meeting. Published by the Network of Aquaculture Centres in Asia-Pacific, Bangkok, Thailand.

Pakingking Jr. R. Aquatic Animal Health Activities of the Fish Health Section, SEAFDEC/AQD. NACA 2016. Fifteenth Meeting of the Asia Regional Advisory Group on Aquatic Animal Health: Report of the Meeting. Published by the Network of Aquaculture Centres in Asia-Pacific, Bangkok, Thailand.

Pakingking Jr. R., de Jesus-Ayson EG (2016) Supporting ASEAN Good Aquaculture Practices: Preventing the Spread of Transboundary Aquatic Animal Diseases. Fish for the People 2: 76-82.

Tendencia EA, VJ Estilo. (2017) Advocating Preventive Measures that Inhibit Early Mortality Syndrome in Shrimps. Fish for the People 15 (3): 30-36; Bangkok Thailand; ISSN 1685-6546.

Tendencia EA, VJ Estilo. (2017) Shrimp survives exposure to low *Vibrio parahaemolyticus* $_{\text{AHPND}}$ load in the water. Poster presentation and Book of Abstract; 10$^{th}$ Symposium on Diseases in Asian Aquaculture

Pakingking Jr. R. (2017) Aquatic Animal Health Activities of the Fish Health Section, SEAFDEC/AQD. NACA 2017. Sixteenth Meeting of the Asia Regional Advisory Group on Aquatic Animal Health: Report of the Meeting. Published by the Network of Aquaculture Centres in Asia-Pacific, Bangkok, Thailand.

Pakingking Jr. R., de Jesus-Ayson EG, Bautista NB (2017) Ocular vibriosis in cage cultured snubnose pompano Trachinotus blochii in the Philippines (Poster Paper Presentation) 10$^{th}$ Symposium on Diseases in Asian Aquaculture, Bali, Indonesia, 28 August to 1 September 2017.

Pakingking Jr.,R, Bautista, NB and de Jesus-Ayson, EG (2018) Characterization of *Vibrio* isolates recovered from the eyes of cage-cultured pompano (*Trachinotus blochii*) infested with caligid parasites (*Lepeophtheirus spinifer*). Bulletin of the European Association of Fish Pathologists, 38 (1): 35-41

Pakingking Jr., Rolando, de Jesus-Ayson, Evelyn Grace, Reyes, Ofelia, Bautista, Norwell Brian (2018) Immunization regimen in Asian sea bass (*Lates calcarifer*) broodfish: a practical strategy to control vertical transmission of nervous necrosis virus during seed production. Vaccine 36: 5002–5009.

Pakingking Jr. R. (2018) Immunization regimen in high value marine broodfish: a pragmatic strategy to control vertical transmission of nervous necrosis virus during seed production (Oral Presentation). Asian Aquaculture 2018: Celebrating Asian Aquaculture, Asian Institute of Technology Convention Center, Klong Luang, Pathumthani, Thailand, 3-6 December 2018

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