Kingdom of Saudi Arabia
Ministry of Agriculture
Deputy of Fisheries Resources Affairs
Department of Aquaculture
Biosecurity Division

MANUAL OF BIOSECURITY AND STANDARD OPERATING PROCEDURES FOR SHRIMP CULTURE

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1. Introduction

The Biosecurity for the shrimp industry in the Kingdom of Saudi Arabia is a priority due to the presence of exotic and endemic diseases, which pose ~~a~~ potential risk for this growing aquaculture industry. The objective of this Manual is to ensure the shrimp aquaculture sustainability by following the procedures and the Standard Operating Procedures (SOP) described in this document.

The main diseases reported in the shrimp industry of the Kingdom of Saudi Arabia are described below:

ADMA listed diseases important for the shrimp culture in the Kingdom of Saudi Arabia

Disease	Listed in the OIE Aquatic Animal Health Code (2014)	List of diseases present in the KSA	Category
1. White spot disease	Yes	Yes	C1
2. Taura syndrome	Yes	Yes	C1
3. Yellowhead disease – Yellowhead virus	Yes	No	C1
4. Infectious hypodermal and hematopoietic necrosis	Yes	Yes	C3
5. Infectious myonecrosis	Yes	No	C1, C2
6. Necrotising hepatopancreatitis	Yes	No	C2
7. Monodon Baculovirus (MBV)	No	Yes	C2, C3
8. Microsporidia	No	Yes	C2, C3
9. Gill-associated virus	No	No	C1, C2
10. Monodon slow growth syndrome	No	No	
11. Acute hepatopancreatic necrosis disease AHPND /(EMS)	No	No	C1
12. EHP			

Categories:
C1: OIE listed disease: Potentially catastrophic, economically significant pathogen.
C2: Poses significant disease/economic threat. Most are OIE listed.
C3: An excludable infectious agent with possible disease or economic impacts.

2. Risk ranking levels

Shrimp aquaculture divisions are ranked by their level of business risk impact

from 1 to 8, starting from the highest risk to the lowest risk.

Priority Level	Business Impact Risk Categories		
	High Risk	Moderate Risk	Low Risk
1	Quarantine	Nurseries	Processing* if effluents and waste are treated (except when importing shrimp from other countries) and lab facilities
2	Broodstock Breeding Center	Intensive grow-out ponds	Transit areas
3	Broodstock production unit	Semi-intensive grow-out ponds	Offices and accommodations
4	Nauplii production unit / Maturation		
5	Larval Rearing		
6	Fresh feeds and live feed		
7	Wastes treatment from processing and lab		
8	Harvest		

*Processing plants should have efficient effluent treatment procedures in place and proper disposal of solids. The release of infectious pathogens during processing poses a significant risk to the surrounding industry. If imported product is process the risk of introducing exotic pathogen is high, the impact may affect the whole regional shrimp industry and therefore, it is an unacceptable risk.

3. Geographical zoning

Based on risk ranking, three zones are established following the same rational:

1. Zone 1: High Risk – Quarantine, Broodstock Breeding Center, Broodstock production units, Maturation, Live Feeds and Larval Rearing.
2. Zone 2: Moderate Risk – Nurseries and Farms.
3. Zone 3: Low Risk – Processing, transit and offices-accommodations

4. Strategy

Biosecurity strategy should be a combination of pathogen exclusion coming from outside, control of pathogen spread within a zone and animal health management. Within a commercial scale there is no such thing as a profitable zero risk approach. A realistic approach is to mitigate the risk with practical measures.

Main risks are related to animals (introduction, movements between zones and harvest), water (incoming water and water management), infrastructure, equipment and tools, which have been in contact with animals and culture water. The efforts (investments) should prioritize these three components.

There is a series of known pathogens that are targeted within this biosecurity strategy (see annexure #1- Major viruses infecting marine shrimp, their tissue distribution and transmission).

5. Biosecurity Requirements

The following table describes the key biosecurity requirements for each risk level and relevant areas.

M: mandatory; **R**: Recommended; **N/A**: not applicable; *: unless massive mortalities;

	High Risk				Moderate Risk		Low Risk	
	Quarantine	Broodstock Breeding Center and broodstock production units	Maturation /Nauplii production unit	Larval culture	Nursery ponds	Semi-intensive/ grow-out ponds	Processing plant & lab services	Site accommodations
Pathogen free status	M	M	M	M	N/A	N/A	N/A	N/A
Inlet water Disinfected	M	M	M	M	M	M	N/A	N/A
RAS	R	R	R	R	R	N/A	N/A	N/A
Indoors	M	M	M	M	R	N/A	N/A	N/A
Covered / enclosed	N/A	N/A	N/A	N/A	R	N/A	N/A	N/A
Effluent treatment	M	N/A	N/A	N/A	N/A*	N/A*	M	N/A
Solid wastes treatment	M	M	M	M	M	M	M	M
Restricted access	M	M	M	M	M	M	M	N/A

The following highlights the specific biosecurity requirements for each risk level and relevant aquaculture activities.

5.1) High Risk

All activities within the high-risk category must be indoors and operated under strict biosecurity measures.

5.1.1 Quarantine

The scope is to validate that animals introduced are free of pathogens (before transferring them to production areas. This is achieved by performing diagnostic analysis for the OIE listed pathogens and other known pathogens. Quarantine is performed indoors; all incoming water is disinfected; all effluents are treated and solid wastes incinerated. (See SOP 2 Quarantine procedures).

The following risk mitigation steps must be followed for the movement of aquatic animals:

- If animals are imported from another country for commercial exploitation, there must be a pre-qualification of the supplier to validate the health status of the animals. Only facilities with animals of documented SPF status will be permitted to export live shrimp into the Kingdom. SPF animals will be submitted to quarantine until their health status is validated as per quarantine protocol. (See SOP 1. proceedings for the importation of live shrimp species into the kingdom of Saudi Arabia)

- If the animals are transferred within Saudi Arabia the SOP regarding movement of shrimp within the KSA must be followed. (See SOP 4 shrimp transfer or movement)

The table below highlights the steps undertaken for the validation of the health status of movements of aquatic animals into and within the Kingdom of Saudi Arabia:

Movements of aquatic animals		From another country	Within Saudi Arabia
Approval Process by ADMA		Pre-qualification of the supplier	Health status validated free of endemic pathogens transfer to the quarantine
		Importation license	Transfer authorization
QUARANTINE	Batch originated from	An ADMA certified supplier	Aquaculture Operation
	1rst Level Quarantine	Required for any SPF new shrimp supplier	N/A
	2nd Level Quarantine	Required for all the broodstock	Required for all the broodstock

5.1.2 Broodstock Breeding Center and Broodstock production units

- The Broodstock Breeding Center and the broodstock production unit are the backbone of shrimp production. The scope is to produce SPF broodstock for maturation and to improve shrimp culture performances through its Family Selection Broodstock Breeding Program.

- Broodstock health is the primary focus of the disease surveillance program (see SOP 18 surveillance). Only animals, which have gone through quarantine or their offspring, can be transferred to the broodstock-breeding center. Broodstock production unit can only accept shrimp that have gone through quarantine or their offspring or, being produced in indoors facilities maintaining the same Biosecurity measures than the Broodstock Breeding Units.

- Production is performed indoors; all incoming water is disinfected; all solid wastes are incinerated. Introduced animals will be tested by PCR on multiple tissues to ensure optimum detection levels (See SOP 14 PCR sampling).

5.1.3 Maturation / Nauplii production facilities

- Nauplii production is performed indoors; all incoming water is disinfected and water temperatures must be kept stable. All wastes are incinerated. Only broodstock originated from the Broodstock Breeding Center or broodstock from other sources but gone through the quarantine process, can enter the maturation.

- Fresh feeds and especially from crustacean origin are a significant biosecurity risk and only artemia biomass is allowed. If outsourced, such feeds must be supplied only from prequalified suppliers. Each batch of artemia biomass must be tested by PCR prior to shipment by a reference lab and confirmed upon reception by the diagnostic lab to be free of WSSV, IHHNV and AHPND. (See SOP 13. Fresh feed and Artemia cysts)

- Dry feeds can act as mechanical carriers but are non-infectious, therefore PCR screening is not necessary, however, they must be free of crustacean by-products.

- Eggs and Nauplii must be disinfected before being transferred (See SOP 18 Surveillance in the shrimp industry and prophylactic measures).

5.1.4 Larval Rearing

- Production is performed indoors; all incoming water is disinfected prior to its use (See SOP 6. Water use in shrimp culture). All solid wastes are incinerated. Only Nauplii originated from the Nauplii Production Unit (maturation) can be stocked in the Larval Production Unit (hatchery). Water temperature should be kept stable.

5.1.5 Fresh feeds and live feeds production

- Artemia hatching is performed indoors; all incoming water is disinfected. All live feeds (algae and artemia cysts, Nauplii and biomass) should be kept free of green TCBS colonies. Enrichment products must be free of shrimp pathogens. Fresh feed used must be originated from countries free of OIE listed diseases for shrimp, and AHPND (EMS).

5.1.6 Wastes treatment from processing and lab services

- Regardless of the origin of raw material the same high biosecurity standard must be maintained. Effluents must be contained, disinfected and disposed without any contact with production areas including farm drainage canals. Solid wastes must be incinerated except for wastes that are further processed through rendering, chitin production, shrimp meal or silage.

5.2) Moderate Risk

Semi intensive grow-out ponds (and intensive grow-out ponds to some extent) are operated outdoor and therefore total exclusion is not achievable. Such production systems require equal emphasis on animal health monitoring, water parameters monitoring and close track keeping of animal performances. All incoming water must be disinfected and filtrated at least preferably at 250 um (See SOP 6 Water use in shrimp culture). Potentially, animals will be exposed to endemic pathogens entering the production system essentially through water or pathogen carriers. Mechanical filtration plays a very significant role but its efficacy is limited due to the large volumes of water needed. This antagonism must be well balanced and every effort must be made to anticipate problems by keeping filtration systems in proper conditions as well as water parameters adequate and stable. In case of symptoms or mortalities linked to a disease outbreak, the contingency protocol must be applied within the shortest possible time frame to minimize the risk of disease spreading (see SOP 8 contingency plan). Stocking and harvesting in blocks is recommended (all in all out strategy). Also, avoid stocking shrimp or, maintain shrimp under culture during the winter season is highly recommended.

5.2.1 Nurseries and intensive grow-out ponds

These structures must be fenced and lined. It is recommended to have these structures covered. Because of high densities and resulting biomasses, animal behavior and animal health must be frequently monitored See SOP 18 surveillance). Since nursery biomasses have no commercial value, in case of detection of pathogen with high economic impact, the biomass must be terminated. In intensive grow-out ponds a disease is likely to develop very fast because of high biomasses, so the detection of a pathogen with severe economic impact must trigger harvest or termination depending on the commercial value of the stock.

5.2.2 Semi-intensive grow-out ponds

These open systems are more vulnerable to water parameter fluctuations, entry of pathogens and spreading of diseases. A strategy aiming at shorter cycles and stocking and harvesting by blocks to minimize the risk is recommended. Animal behavior and animal health must be regularly monitored (see SOP 7 Criteria for shrimp sampling). In case of pathogen detection, it is recommended to harvest or terminate the biomass within the shortest timeframe from the time of pathogen detection. However, the decision should be made from a business risk perspective (See SOP 8 contingency plan).

5.3) Low Risk

Even if a high risk activity such as processing or packaging activities, seafood or live aquatic animal transportation takes place, it does not represent a very significant risk as long as the outcomes from such activities are contained.

5.3.1 Processing Plant

a) Regardless of the origin of raw material, the same high biosecurity standard must be maintained.
b) Vehicles delivering seafood raw material must be disinfected before and after delivery, before loading ice in the case of harvest vehicles. The same applies to the harvest equipment.
c) Effluents must be contained, disinfected and disposed without any contact with production areas including farm drainage canals. Solid wastes must be incinerated except for wastes that are further processed through rendering or chitin production.
d) Imported product cannot be processed as long as the effluent treatment plant has not been efficient.

5.3.2 Laboratory Services

- Movement of laboratory staff, tools and equipment towards production areas should be avoided. It is recommended to deliver the fixed samples to the laboratory.

- Wastes from laboratory services are categorized as high risk (see risk ranking page 2).

5.3.3 Site accommodations and offices

Movement of staff, catering goods and other housing related items are permitted within site accommodations. However, depending on the biosecurity status of the unit, the site access can become restricted.

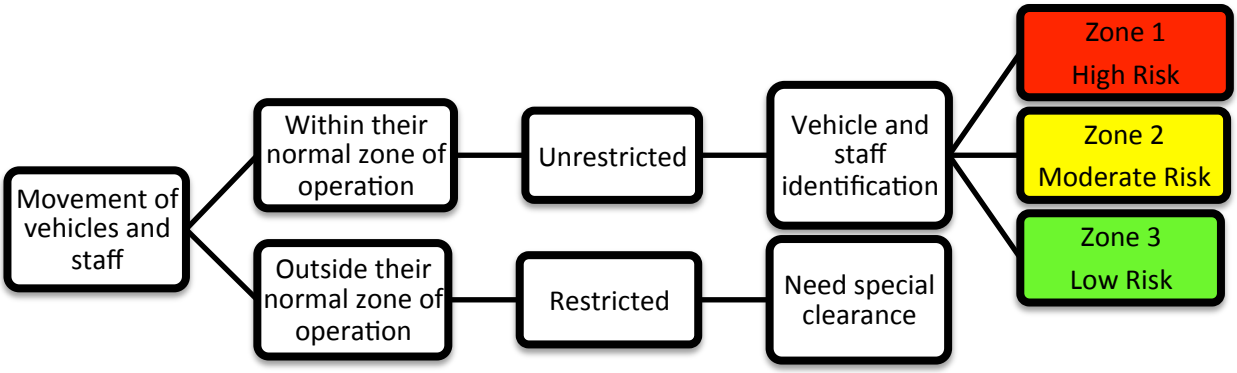
5.3.4 Transit areas

- These buffer zones are used by the different operators, visitors and external services. Movements are subject to biosecurity clearance. Access approval may be restricted depending on the biosecurity status of certain units.

- Biosecurity gates are placed at strategic locations and act as check points to ensure restricted vehicle and staff access.

- Harvest trucks or any other vehicles in case of pond undergoing unusual mortality, must undergo disinfection before leaving the affected area as a risk mitigation measure.

6. Movement of vehicles, staff and shrimp between biosecurity zones:



Drivers are responsible to ensure that people, equipment or goods transported in their vehicles have proper biosecurity clearance.

- Requirements regarding movement of **staff** outside their zone of operation:

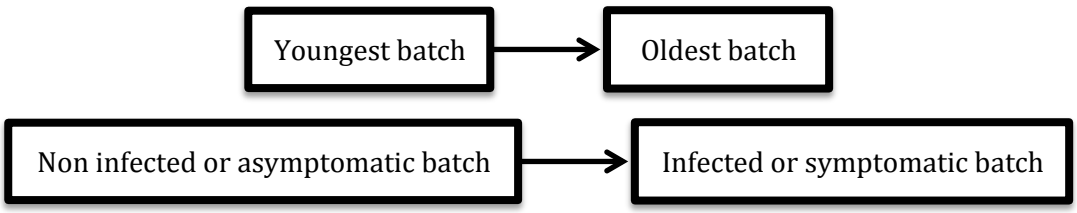
Movement of staff between zones		Access
From	To	
Zone 2	Zone 1	Not allowed
Zone 1	Zone 2	Only for fry delivery and restricted to outside the hatchery
Zone 3	Zone 1 or 2	Only for service purposes
Zone 1 or 2	Zone 3	Unrestricted except to processing plant, laboratory and harvest areas, delivery nauplii, PLs

- Requirements regarding movement of **shrimp** outside their zone of operation:

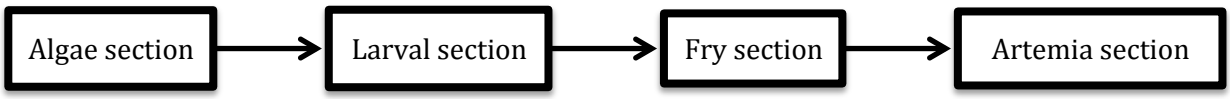
Movement of shrimp between zones		Access
From	To	
Zone 2	Zone 1	Not allowed
Zone 1	Zone 2	Only to stock grow out /nursery ponds
Zone 1	Out of the farm	Allow only if ADMA issue the movement certificate (SOP 3 Certification)

- Requirements regarding movements of **people** within a production unit:

1. All production units



2. Hatcheries



- ✓ **Fishing and other recreational activities:**

  Fishing and other recreational activities (swimming, diving, etc.) involving the use of canals, lagoons and seashore within the site requires authorization. Access to ponds and other production facilities is forbidden for any activity other than related to production. For fishing the use of artificial baits is mandatory.

7. Reporting, Alarming system and Notification

- Any POSITIVE result for WSSV or TSV (by PCR or Histology) or any of the ADMA listed diseases, will be immediately informed to ADMA and the Biosecurity committee. (Immediately means, an email to the ADMA representative must be sent on the same day that a positive result is found.

- ADMA will inform to the different farms about any positive result of any of the ADMA listed diseases.

- All the farms are required authorizing the reference lab to send a copy of the sanitary report to ADMA to compile the whole sanitary information from the KSA shrimp industry

8. Implementation process and auditing

- The responsible person in each farm will be the farm General Manager or his designee.

- The audit process will be a continuous process.

- ADMA representatives will carry out a periodic and/or spontaneous visit.
- A checklist will be filled out to see the effectiveness of the Biosecurity protocol implementation.

- In order to verify implementation of the protocol, the farms will keep the sanitary results in a designated folder and in a database. ADMA representative will verify the records, the notifications of positive results by PCR and the elimination of the ponds (if any) among others.

SOP 1. Proceedings for the importation of live shrimp species into the kingdom of Saudi Arabia

The Aquaculture Division of the Ministry of Agriculture (ADMA) of Saudi Arabia has prepared the Standard Operating Procedures for Importation of live shrimp species into the Kingdom and several Proceedings related to the different activities involved in the process. The aim is to maintain the high sanitary status in the Kingdom, to avoid the introduction and propagation of exotic diseases that could affect the different shrimp species and to serve as a guide to the different shrimp farmers. Each company, interested in importing live shrimp species into the Kingdom must follow this manual and the different Proceedings accompanied.

General Proceedings for the Importation of live shrimp species:

1. Approval of suppliers

Importation of life animals requires approval of supplier, which will imply the visit to the supplier facility, analysis of diagnostic records of at least the last 2 years and records of introductions to the facility during this period.

2. Importation request and documents

The company that is interested in importing live shrimp species into the Kingdom must notify the Aquaculture Division of the Ministry of Agriculture (ADMA) their intention at least 30 days in advance prior to the expected arrival date. The information to be provided through the Request of Importation is shown in Table 1.

Table 1: Request of Importation Document necessary for the Importation of live shrimp species

1	Name of the importing company, address, identification # number, telephone and email ~~of~~ contact
2	Name of the exporting company and country of origin
3	Name of the live shrimp species to be imported (scientific and common names)
4	Number of animals per species to be imported
5	Stage of the live shrimp species to be imported (nauplii, postlarva, juveniles, broodstock)
6	Origin of the water where the live shrimp species to be imported and their parents are / were kept (open system, recirculation system etc.)
7	Expected dates of arrival
8	Port of arrival in the Kingdom of Saudi Arabia (name of sea-port and or air-port)
9	Name of the farm and location where the live shrimp species will be maintained

It is important to note here that the live shrimp species to be imported can ONLY be sourced from sites approved by ADMA. If the live shrimp species is exotic in the Kingdom of Saudi Arabia, and it is the first time that it is imported, an Import Risk Analysis (IRA) must be performed including environmental/ecological, genetics and pathogens risk analysis. While methodology for genetic and ecologic/environmental risk assessments are not codified, a standardized framework for pathogen risk analysis (import risk analysis, IRA) for live aquatic animals and their products is laid out in the World Organization for Animal Health's (OIE) Aquatic Animal Health Code (OIE, 2012) and FAO documents.

3. Health Certificates

Along with the presentation of the Request of Importation document, the interested importing company must present the corresponding Health Certificates signed by the Official Authority of the exporting country. Table 2 shows the Information required for the Health Certificate..

Table 2: Health Certificates information required for the Importation of live shrimp species

1	**Date and place where the Certificate was Issued**
2	Country of origin
3	Official Authority of the Country of Origin
4	Identification of the Exporter
5	Identification of the live shrimp species to be exported
6	Zone or compartment of origin
7	Identification of the farm origin
8	Stage of development at the time of exportation
9	To mention if the live shrimp are coming from a farm or it is a wildlife animal
10	Purpose of the live shrimp importation
11	Mean of transportation (air, sea, terrestrial)
12	Identification, signature and stamp of the Certification Company

The Health Certificate has to state that the consignment satisfies the following requirements:

1. Come from a country which is under veterinary supervision.
2. Come from a farm which is under veterinary supervision.
3. Show no clinical signs on the day of loading.
4. Come from a zone that is free of the ADMA listed diseases (Table 3).
5. The certificate is valid for 10 days from the date of issuing.

Table 3. List of the ADMA diseases for shrimp

Disease	Category
1. White spot disease	C1
2. Taura syndrome disease	C1
3. Yellow head disease – Yellow head virus	C1
4. Infectious hypodermal and hematopoietic necrosis	C1
5. Infectious myonecrosis	C1, C2
6. Necrotizing hepatopancreatitis	C2
7. MonodonBaculovirus (MBV)	C2, C3
8. Microsporidia	C2, C3
9. Gill-associated virus	C1, C2
10. Early Mortality Syndrome (EMS) or Acute hepatopancreatic necrosis disease (AHPND)	C1
11.Hepatopancreatric microsporidia	C2

The verification of the sanitary status of the shrimp stock to be exported requires that the exporting facility send the samples for sanitary analysis in the reference laboratories for shrimp diseases (See annex 1).

Any Company interested in the importation of any live shrimp species into the Kingdom must have an Authorization provided by ADMA, that allows them to proceed prior to importation. Only importation from ADMA approved suppliers will be considered. At least 72 hours before the expected arrival of the live shrimp species, the importer must show the documents present in table 3 along with the corresponding Health Certificates. These documents must be presented to the ADMA officer located both at the entrance place and also at the division where the species will be allocated.

4. Certificate of origin

The Certificate of Origin is a document that is used in International trade, where the concept of origin refers to the country where the goods were produced and not necessarily originated from. Each shrimp consignment must come with the Certificate of Origin.

5. Additional Importation documents required

Besides the request of Importation and the health certificates, the documents stated in the table 4 are required by ADMA to issue the authorization for importation:

Table 4: Additional Documents requested during the process of Importation of live shrimp species

1	Copy of the Invoice provided by the Exporter
2	ADMA approval certificate
3	Air bill tracking document – number
4	Import Risk Analysis (IRA) in the case of an importation of Exotic – non native species
5	Request of Quarantine (SOP 2)
6	ADMA Quarantine Certificate authorizing the unit where the consignment will be allocated (SOP 2)

- All these documents will be submitted to ADMA. In a time frame of 1 month, ADMA will study the documents and will issue the authorization for importation.

- In case that the species to be imported is considered as endangered species according to CITES (Convention on International Trade in Endangered species of Wild Fauna and Flora), ADMA needs to approve the sanitary status. In this case the office from the exporting country and the importing countries will issue the authorization for exporting and importing respectively.

- Once the shrimp enter the KSA, no opening of the boxes is allowed until the final destination, where proper disinfection and elimination of the boxes, water and ice is done.
  All shrimp in the consignment must be packaged in leak-proof bags, each bag containing only one species. The bag must be colorless and sufficiently transparent to enable proper inspection and identification of the shrimp and must not contain any extraneous matter, unapproved plant material or pests.

- The consignment must be accompanied by documents that include the identification number of each box or carton, and the scientific name and number of the contained shrimp. It is recommended that the common names of the shrimp also be included on the papers.
- All shipments of shrimp will be inspected by ADMA on arrival to ensure that they:
  1. Are healthy
  2. The veterinary certification and invoice is in order
  3. Are an approved species
  4. Do not contain prohibited material or material of quarantine concern.

- Shrimp not meeting these criteria and prohibited material will be seized or exported or destroyed at the importer's expense
- All shrimp will be ordered into quarantine at an ADMA approved location (See SOP 2 Quarantine procedures).

SOP 2. Quarantine procedures

Movement of shrimp from abroad

- Only shrimp population with ADMA approval from the exporting country will be allowed to enter the primary quarantine facilities.

- A primary quarantine is the one to receive the animals from abroad and will hold them until all the required testing has shown not to pose an infectious risk. A primary quarantine is used for the first introduction of a particular supplier. A secondary quarantine is a private sector quarantine that needs approval from ADMA before the reception of animals released from the primary quarantine. Based on ADMA approval, a primary quarantine may be a government or private sector facility. In case that the primary quarantine is a private sector facility, a secondary quarantine might not be required.

First level quarantine facilities for imported live shrimp from an ADMA certified supplier

1. A request for quarantine must be filled up and sent to ADMA for approval.

2. It is recommended to be adequately isolated from all of the rearing and production areas to avoid any possible cross contamination.

3. It must be in an enclosed and **covered** building facility.

4. There must be means provided for disinfection of feet (foot-dip deep containing hypochlorite solution at 50 ppm active ingredient) and hands wash facility (bottles containing 70% alcohol / or iodine solution at 100 ppm) to be used upon entering and exiting the unit.

5. Entrance to the quarantine area must be restricted to the personnel assigned to work exclusively in this area.

6. Quarantine unit staff must enter through a dressing room, where they remove their street clothes, take a shower and put on working clothes and boots specific for the quarantine. At the end of the working shift, the sequence is reversed.

7. Pumped water will go through a mechanical filtration including sand filter to obtain the water filtered to at least 1 um.

8. Water disinfection with either 15 ppm (72 h),30 ppm chlorine (24 h) or ozone at 0.5 mg/L (10 minutes contact time; 8 minutes for 1.5 ppm) any other disinfectant to ensure proper water disinfection would be used and

must to be validated by bacteriology in a general media.

9. Water will pass through activated charcoal unit.

10. Water will pass through UV light at 10 ml/cm 2.

11. All the tanks must be washed with soap and water and disinfected with hypochlorite solution (100 ppm active ingredient) and rinsed with disinfected water.

12. All wastewater must be collected for chlorination (100 ppm for not less than 1 day) and de-chlorination before released to the environment.

13. Used plastic containers and hoses must be washed and disinfected with hypochlorite solution (100 ppm) or other disinfectants at an equivalent concentration, before reuse.

14. All the materials used in the quarantine unit must be clearly marked and should remain in the quarantine area. Facilities for disinfection of all equipment at the end of each day should be available.

15. On entering the quarantine area, the shrimp should be gradually acclimatized to the same temperature and salinity of the tanks.

16. Once the shrimp are placed in the holding tanks, the packing plastic bags, boxes, and any other disposable material related to the shrimp packing must be incinerated. Styrofoam boxes could be disinfected at 200ppm chlorine and allow to dry for 5 days.

17. Dead shrimp will be sampled for PCR of ADMA listed pathogens. For a first importation all ADMA listed pathogens must be tested. Gills/pleopods organ and hepatopancreas from each recent dead shrimp will be individually preserved in 95% ethanol (See SOP 14 Sampling for PCR analysis).

18. Any symptomatic shrimp will be fixed in Davidson's fixative by injection, maintained for 48 h and then transferred to a 70% ethanol plastic container for histological analysis (see SOP 14 for fixation).

19. When the shipment involves low temperature (animals have been packed and shipped at temperatures <=22C), it is assume that no further cold challenge is needed. If this situation does not happen, a cold challenge is required: 10 random shrimp will be individually passed through a dip of iodine-PVP solution (20 ppm for 10" minutes) or formalin (100 ppm) and placed in the cold challenge facilities (See SOP 12 Cold challenge).

20. **Cohabitation studies** are required for a first introduction of a new species or new supplier: After acclimation, 5-10 random shrimp are taken and placed them in a different tank along with 10 healthy *P. indicus* from the Fish Research Center (JFRC) and 5 SPF *P. vannamei* of about the same weight (with respective controls for each species). The *P. indicus* used, must have a history of freedom of any of the OIE and ADMA listed pathogens and no unusual mortalities (this may pose a problem depending on the health status of the local *P. indicus* stocks).

21. **Ingestion challenge** is required for a first introduction of a new species or new supplier: After acclimation, 5-10 random shrimp are taken from the quarantine and homogenized them by grinding the whole tissue to feed 10 healthy *P. indicus* from the Fish Research Center (JFRC) and 10 SPF *P. vannamei*. Shrimp will be fed with the 3% of biomass/day in two rations (AM, PM) for three days. The *P. indicus* used, must have a history of freedom of any of the OIE and ADMA listed diseases and no unusual mortalities (this may pose a problem depending on the health status of the local *P. indicus* stocks).

22. If presence of shrimp displaying any clinical signs during the cold, cohabitation or the ingestion challenges, shrimp samples will be immediately collected for histological and PCR analysis.

23. After 5 days of cold challenge, every shrimp will be individually sampled. Samples of gills and hepatopancreas will be taken and preserved in 95% ethanol for PCR analysis. 5 shrimp will be fixed for histological analysis (See SOP 15 fixation of shrimp and crustacean samples in Davidson's fixative).

24. After 10 days of **cohabitation challenge**, every *P. indicus* and *P. vannamei* will be individually sampled. Samples of gills and hepatopancreas will be taken and preserved in 95% ethanol for PCR analysis. 5 shrimp from each species will be fixed for histological analysis (See SOP 15 fixation of shrimp and crustacean samples in Davidson's fixative).

25. After 10 days of **the ingestion challenge**, every *P. indicus* and *P. vannamei* will be individually sampled. Samples of gills and hepatopancreas will be taken and preserved in 95% ethanol for PCR analysis. 5 shrimp from each species will be fixed for histological analysis (See SOP 15 fixation of shrimp and crustacean samples in Davidson's fixative).

26. Samples of a first introduction must be sent to an international reference lab (according to OIE) for histological analysis and PCR analysis for WSSV, TSV, YHV, IMNV, MBV, IHHNV, AHPND and NHP-B (See annex 1

Reference labs). Subsequent shrimp introductions can be analyzed at the national reference lab or ADMA approved laboratories.

27. In the case that any sample gives a positive result for any of the ADMA listed diseases (See table 3) or display histopathological changes which are not recognized and might pose concern, the whole population will be discarded.

28. Only shrimp stock free of any of the ADMA listed diseases by PCR and histology would be taken to the secondary quarantine.

Second level quarantine

- This quarantine has the same requirements in terms of infrastructure and water treatments as a primary quarantine. The second level quarantine could be a private sector quarantine approved by ADMA that could maintain shrimp if the following requirements are met:

  1. Shrimp SPF stock from a primary quarantine that met all the health sanitary status required by ADMA.

  2. Shrimp SPF stock from an approved secondary quarantine.

- In secondary quarantine, samples for PCR and histology analysis will be taken and only if the animals are free of the ADMA listed disease, the stock would go to the production area. The check list for the quarantine is described below:

Kingdom of Saudi Arabia
Ministry of Agriculture
Department of Aquaculture, ADMA
Biosecurity Division

Fish Facility:
Date:
Location:

Check list for Quarantine Unit approval

Characteristic	Result	Notes
1- Identification of the name of the company and the specific farm, plus the location and contact information.		
2- Present a general design together with the description of the specific technical characteristics of each part of the unit, including drawing and maps.		
3- Must be enclosed and covered with no direct access to/from outside.		
4- has an exclusive entrance, restricted only to authorized people who are working – also exclusively- in the unit.		
5- Must be separated and isolated from any other farming operation.		
6- Has record systems of all activities and management.		
7- Has an independent entrance, circulation – flow and exit of the water.		
8- Has system of water recirculation.		
9- Has a water treatment system that include filtration of the discharged water.		
10- Has a water treatment system that include disinfection of the discharged water.		
11- Has specific – properly identified areas for disinfection of people and materials at both the entrance and exit of the unit.		
12- Implements cleaning, aeration and disinfection system in all of the areas.		
13- Has special clothes and boots that are used exclusively inside the unit.		
14- Has an independent - physically separated room, for the inspection and sample preparation for the pathology test. This room is located inside the Quarantine Unit, but separated from where the animals are kept.		
15- Has proper proceedings and infrastructures for the destruction of the used materials and mortalities.		
16- Standard Operating Procedures are present and applied in the unit facilities.		

ADMA Name and Signature:

Contact person Name and Signature:
Request of Quarantine

Approval
#_____

Name of Importer			
Name and Location of Quarantine Unit			
Initial date		**# animals**	
Finish date		**# Invoice**	
Address		**Phone**	

Item	**Common name and sex**	**Scientific name**	**Quantity**	**Inspection**	
				1	**2**
1					
2					
3					
4					
5					
6					
7					
8					
9					
10					
Total					

Observations

Date of Inspection
1
2

Date of Quarantine start
Name and signature of Inspector **Official Stamp**
Quarantine extension

Details

Date of Quarantine release
Name and signature of Inspector **Official Stamp**

Quarantine Records

Mortality Records

Approval #_____

Days	Item					Observations
	1	2	3	4	5	
1						
2						
3						
4						
5						
6						
7						
8						
9						
10						
11						
12						
13						
14						
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22						
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24						
25						
26						
27						
28						
29						
30						
31						

Name and signature of Quarantine Unit responsible

SOP 3. Certification of shrimp movement within the country

In order for ADMA to issue a Movement certificate, the necessary information must be provided:

1. Written Request of shrimp movement 5days before the movement.

2. Include the following information:

    a) Description of to/from the shrimp will be moved
    b) Date of the movement
    c) Species and stage
    d) Origin of the stock

3. PCR results for endemic pathogens from an ADMA approved lab. Only stocks with negative results for endemic pathogens would be allowed to be moved.

SOP 4. Shrimp transfer / Movements

1. Transfer of broodstock

- Non-SPF stocks raised in the same facility have to be directed to Quarantine (SOP 2 Quarantine procedures).

- SPF stocks transferred within the same operation, while recommended, do not require further testing.

- SPF stocks transferred to another operation will require PCR testing for endemic pathogens and histology analysis.

2. Transfer of nauplii

- Nauplii originated from SPF stocks need no certification for movement within the same facility.

- Nauplii that are transferred to another operation will only be allowed from broodstock with proven sanitary records.

3. Transfer of PLs

- PLs will require cold challenge (SOP 12 Cold challenge) and PCR certification for endemic pathogens before transferring to nurseries or grow-out ponds.

- Only stocks with negative results for endemic pathogens would be allowed to be moved.

4. Transfer of juveniles

- Juveniles in nurseries that have maintained at the same biosecurity level as in larval rearing will not require PCR testing when transferred within the same operation.

- If juveniles have already been exposed to a lower biosecurity level and therefore, PCR testing for endemic pathogens is required prior transfer to a pond even within the same facility.

SOP 5. Use of wild shrimp in aquaculture facilities

1. It is prohibited to use wild shrimp for direct aquaculture production.
2. Only hatchery produced animals can be used to stock aquaculture ponds.
3. Wild animals may be used for specific purposes (i.e. increase genetic diversity in breeding programs). In that case, animals must be subjected to Quarantine procedures (SOP 2 Quarantine procedures).
4. Prior to collection, ADMA must approve the collection of wild animals and must approve the quarantine facility to be used.
5. Wild animals to be used in a prawn hatchery must be subject to the following quarantine procedures:
    a) All animals brought from the wild must be maintained in the quarantine facilities in individual quarantine tanks.
    b) All animals brought from the wild must be tested individually by PCR for all ADMA listed shrimp pathogens.
    c) Target tissues to be analyzed are pleopods/gills and feces depending on the target pathogen.
    d) Any animal that tests positive, to any ADMA listed diseases must be destroyed.
    e) Animals that are negative to all ADMA listed diseases may be pooled together.
    f) Within the quarantine facility, the wild broodstock (negative to ADMA listed diseases may be used for production of offspring.
    g) Females will be tested a second time after ablation. After the spawning, both male and female will be sacrificed and analyzed by histology. If positive for any ADMA pathogen or unknown pathogen, offspring must be discarded. The remaining tissues/organs must go through histology and they need to be cleared by a veterinarian or pathologist.
    h) These offspring (First Generation) must be reared in a separate wing within quarantine facility and tested at 10 grams for all ADMA listed prawn diseases.
    i) Any batch that test positive, to any ADMA listed diseases, must be destroyed.
    j) Based on the sanitary data, ADMA would approve the introduction of the shrimp population into the Aquaculture facilities.

SOP 6. Water use in the shrimp culture

1. Breeding center, indoors broodstock, Nauplii production units and Larval rearing units

1.1 Pumped water will go through a mechanical filtration including sand filter to obtain the water filtered to at least 1 um.

1.2 Water disinfection with either 15 ppm (72 h),30 ppm chlorine (24 h) or ozone at 0.5 mg/L (10 minutes contact time; 8 minutes for 1.5 ppm) any other disinfectant to ensure proper water disinfection would be used and must to be validated by bacteriology in a general media.

1.3 Water will pass through activated charcoal unit.

1.4 Water will pass through UV light at 10 ml/cm ^{2}.

1.5 It is recommended that discharged water be retained in effluent treatment ponds especially in cases of disease outbreak.

1.6 All solid wastes (dead animals, molts, food left over, etc.) recovered must be disposed off properly (incineration or burial).

1.7 There will be appropriate documentation of each step in the process using the formats provided. In particular there must be clear documentation of ozone and/or chlorination procedures (see SOP 17 Disinfectants) of incoming water. Where chlorine is used as a disinfectant, the activity of the product will be tested and that value used in the calculation of dosing rates. Additionally there must be clearly documented evidence of sustained free chlorine availability in water held during the 72-hour period. When ozone is used as the disinfectant of choice there will be documentation of Oxidation Reduction Potential (ORP) levels.

2. Nurseries and outdoor ponds

2.1 Filter screens including the wooden water level control boards (known as slabs) are fixed in the main water supply canal. Generally, double layer filters (1,000 and 500µ) are used in large water control structures, like main and secondary reservoir channels. Main water supply canal is filtered initially with 1,000µ and installed additionally with 250µm bagnets.

2.2 All pond water-filling requirements need to be checked by responsible

managers before proceeding to next activity. When desired water level is achieved, ensure that inlet and outlet gates are soil-sealed before applying ADMA approved crusticide (0.5ppm) and copper sulphate (0.5ppm). Copper sulphate is used to eliminate zooplankton which is known to be carriers of WSSV and which may be transmitted to shrimp. Secondary water supply canal is also disinfected with same concentration of ADMA approved crusticide and copper sulphate. Chlorination is another option and is widely used to disinfect production or culture ponds in preparation for post larvae stocking. The common dose used for killing disease vectors and for treating pond water requires 30ppm active ingredient in the water.

2.3 After application, filter screen with 250µ mesh can be fixed to each pond inlet gate only when desired water depth in a pond is achieved and when final disinfection is completed and as such is only used for water exchange. It is worth remembering that treatment has to be done as effectively as possible as chemicals represent an import part of the cost of production in semi-intensive culture.

2.4 All screens and control boards should be inspected regularly for leaks and all leaks must be sealed to prevent unfiltered water from entering ponds using rubber gasket type material. No debris recovered from screens or bag nets or other material is to be put back into supply canal or pond. Put all this material in buckets and dispose of in designated pit and cover it with hydrated lime.

2.5 It is recommended to take plankton samples of the reservoir ponds/tanks for WSSV by PCR 3 days after the treatment.

SOP 7. Criteria for shrimp sampling in ponds/tanks for sanitary analysis

1. Start the surveillance in the healthier ponds/tanks in order to avoid cross contamination from a symptomatic pond to a healthy pond/tank. This could be based on the following variables. Start with ponds that have:

    a) High survival rate
    b) No history of mortalities
    c) No birds presence
    d) New ponds

2. The last pond/tank sampled must be the ones that have reported mortality.

3. If a pond/tank displaying mortality is sampled, do not take samples in another pond/tank unless some mortality is observed as well.

Recommended procedure to increase the chance to take a shrimp displaying clinical signs

Grow out pond:

1. Let the selected pond drain for at least 20 min.

2. Take the sick shrimp adhered to the outlet gate net.

3. If no sick shrimp observed in the outlet net, take the samples in that area of the pond by cast netting.

4. Place the shrimp in a clear bucket to observe unusual discoloration.

5. If shrimp looks moribund in the bucket, select them for sampling.

6. Select preferably shrimp displaying any clinical signs such as:

    a) Moribund
    b) Dark /red discoloration
    c) Melanization or dark spots in the exoskeleton
    d) Soft shell
    e) Weak behavior
    f) Black gills

7. Take samples for PCR and histology according SOP 14 and SOP 15.

Tanks:

1. Cut the air flow.

2. Select preferably shrimp displaying any clinical signs such as:

    a) Moribund
    b) Dark /red discoloration
    c) Melanization or dark spots in the exoskeleton
    d) Soft shell
    e) Weak behavior
    f) Black gills

3. Take samples for PCR and histology according SOP 14 and SOP 15.

SOP 8. Contingency plan

Upon presence of any of the ADMA listed diseases in the category C1 in Quarantine, breeding program, commercial broodstock, postlarva and nursery or massive mortality in grow-out ponds, the affected stock will be harvested or eliminated depending on the economical value. See table below.

Pathogen	Category	Quarantine	Breeding program	Commercial broodstock	Post larvae & nursery	Grow out
High risk						
EMS	C1	Eliminate	Cleaning	Eliminate	Eliminate	Harvest/eliminate
WSSV	C1	Eliminate	Cleaning	Eliminate	Eliminate	AM Temperature ≥30ºC: Monitor*
						AM Temperature ≤30ºC:: Harvest /Eliminate
IMNV	C1	Eliminate	Cleaning	Eliminate	Eliminate	Harvest/eliminate
TSV	C1	Eliminate	Cleaning	Eliminate	Eliminate	Harvest/eliminate
YHV	C1	Eliminate	Cleaning	Eliminate	Eliminate	Harvest/eliminate
Moderate risk						
EHP	C2	Eliminate	Cleaning	Eliminate	Eliminate	Eliminate /harvest if severe
NHP	C2	Eliminate	Cleaning	Eliminate	Eliminate	Eliminate /harvest if severe
Low risk						
IHHNV	C3	Eliminate	Cleaning	Individual selection	-	-
BP	C3	Individual selection	Cleaning	Individual selection	Eliminate if severe	-
MBV	C3	Individual selection	Cleaning	Individual selection	Eliminate if severe	-

* Increase monitoring of Animal Health and mortality on affected ponds kept in production

* Improve management of ponds to minimize stress that may trigger an increase in mortalities

a. *Increase aeration

b. *Increase water exchange to improve water quality

SOP 9. Emergency harvest or termination of a pond

Procedure:

1. Upon presence of massive mortality from any of the ADMA listed diseases in the category C1 in a shrimp pond, the affected stock will be harvested or eliminated depending on the economical value
2. Harvest or termination should be done within the shortest possible time frame.
3. Termination will be carried out by applying ADMA approved crusticide with close gates and a 10-12 days retention time. After that, water can be drained safely and dead shrimp needs to be collected and disposed off appropriately (incineration or burial)
4. Harvest of these ponds should be prioritized over any other harvest and it should start as soon as water levels are appropriate.
5. Place a net of a mesh in the discharge canal that can contain any shrimp escape from the harvesting process.
6. Shrimp harvested will be taken to the processing plant avoiding to spill water
7. Apply the SOP 9 and SOP 10
8. All the material will be disinfected with chlorine at 100 ppm or any other disinfectant (SOP 17) and sun dry.
9. Personnel involved in the harvesting process will change their clothes and boots in order before continuing with their taks.
10. Vehicles involved in the emergency harvest will be washed and disinfected (SOP 17).

SOP 10. Crustacean eradication during pond preparation

Procedure:

1. After a pond harvested or terminated, crustaceans in the pond bottom need to be eradicated before stocking new shrimp.

2. All staff involved in handling chemicals must be wearing adequate protection equipment.

3. Fill up the pond with water to reach about 30 cm of water column or enough water to ensure the entire pond surface is covered.

4. Gates must be previously sealed.

5. Doses depending on the product applied:

Chemical	Doses	Application
ADMA approved crusticide	It will depends on the active ingredient (see SOP 17).	Unique application, retain the water for at least 5 days (It will depends on the chemical).
Calcium hypochlorite	50 ppm	Apply during the afternoon and re-enforce daily for 5 days.

6. After 10-12 days, drain the pond and with a shovel make a hole of 20 cm in different areas of the pond.

7. Check for live crustaceans. If present, repeat the steps 3 through 5.

SOP 11. Termination of a shrimp tank

1. Upon presence of massive mortality or positive PCR from any of the OIE listed diseases in the category C1, the affected tank will be immediately terminated.

2. All staff involved in handling the pesticides/disinfectant must be wearing adequate protection equipment.

3. Stop any water exchange and close the drainage pipes and seal the outlets.

4. Discard the tank by chlorinating at 100 ppm for 24 h. or by applying an equivalent crusticide (dosage based on table on SOP 17 Disinfectants).

5. All the materials/equipment that have been in contact with the shrimp and water must be disinfected by chlorination at 100 ppm, rinsed with water and sun-dried properly before storage.

6. This remains restricted area, sealed for any visit for 1 day.

7. After 1 day, tank water can be drained out if no chlorine residues.

8. Use bag nets when the tank is drained out to retain the dead shrimp.

9. Ideally, dead shrimp must be incinerated. Burial is also an acceptable disposal method.

10. Bags with dead shrimp must be transported to an identified pit area.

11. The entire surface of the pit should be covered with a layer of soil of at least 10cm.

12. Disinfection by chlorination of the water pipes must be done with 100 ppm of Chlorine for 24 h.

SOP 12. Cold challenge

1. Cold challenge methodology allows the replication of certain viruses, which results in a higher chance of detection of low level of infections. This procedure is required for any shrimp that go through a primary quarantine. Also, it is required for non-SPF broodstock, and different stages of animals, originated from SPF stock that have been exposed to lower levels of biosecurity.

2. The cold challenge room must be adequately isolated from all of the rearing and production areas to avoid any possible cross contamination.

3. There should be means provided for disinfection of feet (foot dip deep containing hypochlorite solution at 50 ppm active ingredient) and hands wash (bottles containing 70% alcohol / or iodine solution at 100 ppm) to be used upon entering and exiting the unit.

4. Tanks will be filled up with seawater previously chlorinated at 30ppm. Then if residual chlorine is present, sodium thiosulfate will be added or strong aeration.
5. Adjust water temperature 22ºC +/- 2ºC.

6. Recommended densities for the challenge:
   - 25 Postlarva /L
   - 4 juvenile/L
   - 0.5 broodstock /L

7. 150 Pls will be used for a challenge test (2% expected prevalence) for 48 h.

8. 150 juveniles and broodstock will be the cold challenged for 5 days.

9. The effluent water will be discarded previous chlorine disinfection at 100 ppm.

10. Any dead shrimp gathered during cold challenge will be sampled. Tissues will be taken for WSSV, IHHNV and TSV analysis by PCR (See SOP 14 Sampling for PCR analysis).

11. The 150 Pls without the eye-stalk will be pooled in one sample for PCR analysis.

12. Juveniles and broodstock tissue such as pleopods, gills, lymphoid organ or hemolymph, will be taken and 10 subsamples of 15 pieces each (total 150 shrimp) will be pooled and analyzed for WSSV, IHHNV and TSV by PCR. See SOP 14 (Sampling for PCR analysis).

13. In the case of broodstock, if clinical signs are displayed during the challenge 10 affected shrimp in Davidson for histological analysis (See SOP 15 Fixation of shrimp and crustacean samples in Davidson's fixative).

14. Equipment used for transferring or disposing the animals must be disinfected by dipping it in 100 ppm sodium hypochlorite and rinsing with clean sea water and drying.

15. All the materials used for the cold challenge must remain in that place.

SOP 13. Fresh feed and artemia cysts

1. Fresh feeds are a significant biosecurity risk. In the shrimp industry polychaete, squid, clams and artemia biomass are the most used in maturation / Nauplii Production Units. Supplies cannot come from geographical areas where there are endemic diseases of significant commercial importance (ADMA listed diseases).

2. Fresh feed and artemia cysts suppliers must provide a certificate of origin and disease free status from an OIE reference laboratory.

3. Crabs or shrimp or any decapod cannot be used as a fresh feed.

4. These two conditions must be a pre-requisite for obtaining the importation license.

5. Artemia biomass must be analyzed by PCR for WSSV and IHHNV.

SOP 14. Sampling for PCR analysis

In order to protect the nucleic acid (DNA, RNA) for PCR, RT-PCR analysis, samples need to be preserved under proper conditions such:

- **DNA**: Frozen, 95% ethanol.
- **RNA**: RNA Later, 95% ethanol, frozen.

Procedure:

1. Samples for WSSV, YHV, IHHNV and TSV by PCR and any other etiology that infect cuticular epithelium, subcuticular epithelium and connective tissue will be:

    a) Lethal samples: pleopods, gills, Lymphoid organ, hemolymph and PLs without eye-stalk
    b) Non lethalsamples: pleopods and hemolymph

2. Samples for IMNV by PCR any other etiology that infect striated muscle will be:

    a) Lethal samples: pleopods, abdominal muscle, lymphoid organ, hemolymph and PLs without eye-stalk.
    b) Non lethal samples: pleopods and hemolymph

3. For hepatopancreatic viruses (MBV, BP, BMNV and HPV), bacteria (NHP), and HP microsporidia a piece of the hepatopancreatic lobule is collected.

    a) Lethal samples: Hepatopancreas
    b) Non lethal samples: Feces

4. Samples for the Acute Hepatopancreatitis Necrotizing disease AHPND (EMS) could be pre-grown in a 1.5%NaCl containing TSB (trypticase Soy Broth) at 30ºC for 4 h in shaking conditions. If use of AP4 primers for AHPND PCR, this step could be omitted.

    a) Lethal samples: hepatopancreas, stomach
    b) Non lethal samples: Feces

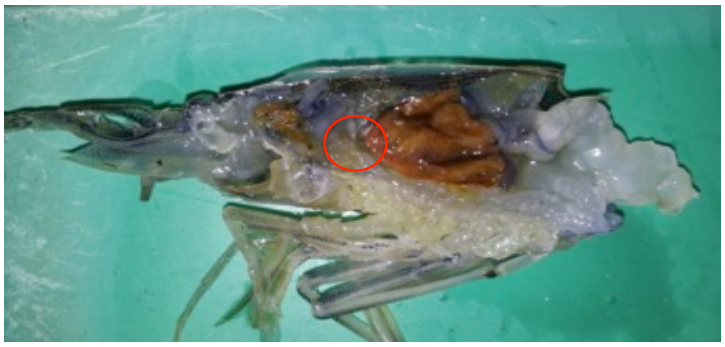
Lymphoid organ sampling (only broodstock):

Shrimp cephalothorax will be cut longitudinally in two halves (see picture).

1. Use sterile scissors and forceps to take the samples.

2. Pass the forceps/scissors by 70% alcohol and flame them before taking any other sample.

3. Area in front of the hepatopancreas (see red circle) will be taken with a forceps and placed in a 1 ml micro tube previously filled up with 95% ethanol.



Lymphoid organ location (red circle)

Hemolymph sampling:

Hemolymphwill be withdrawn from the ventral area of the abdomen just close the junction to the cephalothorax (see picture). For this purpose, a 1 ml insulin syringe will be used.

1. Immediately the hemolymph is withdrawn, place it in a 1.5 ml micro tube containing 95% ethanol. The process needs to be done fast to avoid the hemolymph to clot.

2. The syringe and needle must be used only once.

Gills and pleopods sampling:

Pull out with a sterile forceps some gills lamella / pleopods and place them in a 1.5 ml micro tube containing 95% ethanol. Depend of the number of samples, a 15 ml tubes can be used.

1. Between samples, the forceps must be sterilized by dipping in ethanol and flamed.

2. Make sure that the whole tissue is immersed in 95% ethanol ration 1:10 (tissue: ethanol).

3. Label the micro tubes with pencil#2 with the details about the sample.

4. Micro tubes will be covered with parafilm plastic to avoid any leaking.

Back up samples:

Back up samples are samples taken from the **same** shrimp. e**.g.** If a pool of left pleopods / gill / lymphoid organ are used for PCR analysis. Tissue from the same organ must be taken as a back-up sample; i.e. if pleopods is chosen, back-up sample must be a piece of the same pleopods.

1. For back up sample of hemolymph, only half of the hemolymph withdrawn in the syringe will be placed in the sample tube and the other half will be placed in the back up sample tube.

2. In case that some positive results for any of the OIE listed diseases are found in a given sample, the back up sample will be ready to send to the diagnosis lab for confirmation purposes.

3. The backup sample will have the same codification that the sample tubes.

SOP 15. Fixation of shrimp and crustacean samples in Davidson's fixative

Davidson's AFA (alcohol, formalin, acetic acid) fixative. Davidson's AFA fixative is recommended for most histological applications. The fixative is rapid, reduces autolytic changes in tropical crustaceans (i.e. the penaeid shrimp), and its acidic content decalcifies the cuticle.

The formulation for Davidson's AFA is (for 1 liter):

1. 330 ml 95% ethyl alcohol
2. 220 ml 100% formalin (a saturated 37–39% aqueous solution of formaldehyde gas)
3. 115 ml glacial acetic acid
4. 335 ml tap water (for marine crustaceans, sea water may be substituted)

Store the fixative in glass or plastic bottles with secure caps at room temperature.

For larvae and postlarvae (PLs):

1. PLs that are too small to be easily injected with fixative using a tuberculin syringe: Using a fine mesh screen or a Pasteur pipette, select and collect specimens.

2. Immerse shrimp selected for sampling directly in the fixative. Fix for 12–24 hours in fixative, and then transfer to 50–70% ethyl alcohol for storage.

For larger postlarva, juveniles, and adults:

1. Inject Davidson fixative (use 1:1 volume (ml): weight (g) via needle and syringe

2. The hepatopancreas (HP) should be injected first and at two or more sites, with a volume sufficient to change the HP to a white to orange color; then inject fixative into adjacent regions of the cephalothorax, into the anterior abdominal region, and into the posterior abdominal region.

3. Immediately following the injection, cut the specimen after the first abdominal segment.

4. Cut the cuticle in the cephalothoraxic region, with dissecting scissors. The incision should be done just lateral to the dorsal midline.

5. Immerse the specimen in the fixative (use 10:1 fixative: tissue ratio).

6. Allow fixation to proceed at room temperature for 24–72 hours depending on

the size of shrimp (or crustacean) being preserved. Longer fixation times in Davidson's AFA may be used to thoroughly decalcify the shell of crabs, lobsters, crayfish, etc.

7. Following fixation, the specimens should be rinsed with tap water and transferred to 70% ethanol, where they can be stored for an indefinite period.

8. Record a complete history of the specimens at the time of collection: gross observations on the condition of the shrimp (or other crustacean), species, age, weight, source (wild, or if culture pond or tank number, stock number, etc.), and any other pertinent information that may be needed at a later time.

9. The label should stay with the specimens in the same container during fixation, storage and transport to the laboratory. Always use No. 2 soft-lead pencil on water-resistant paper (plastic paper is recommended; never use ink or marking pens as the ink is dissolved by alcohol).

Transport and shipment of preserved samples

Because large volumes of alcohol should not be posted or shipped, the following methods are recommended:

1. Remove the specimens from the 70% ethyl alcohol. For larvae, postlarvae, or small juveniles, use leak-proof, screw-cap plastic vials if available; if glass vials must be used, pack to prevent breakage.
2. For larger specimens, wrap samples with white paper towels to completely cover (do not use raw cotton). Place towel-wrapped specimens in a sealable plastic bag and saturate with 70% ethyl alcohol. Insert the label and seal the bag.
3. Place the bag within a second sealable bag. Multiple small sealable bags can again be placed within a sturdy, crush-proof appropriately labeled container for shipment.

SOP 16. Plankton sampling for PCR analysis

1. Samples of plankton will be taken with a 60 µm net (see the picture below).

2. The net will be introduced in the water (above 30 cm below the water surface) and by boating or walking for 20 minutes by the border line, the sample will be collected in the collector bottles present in the net.

3. The samples will be extracted from the collector bottles with a sterile spatula and preserved in 95% ethanol in micro tubes.

4. Depend of the amount of plankton sample obtained from the sampling; a single sample will be conformed for no more than 1 g plankton sample.

SOP 17. Disinfectants

A key element of disinfection is a choice of a suitable disinfecting agent. Disinfecting agents are selected according to the nature of target pathogen.

For disinfection purposes virus falls into three basics groups

Category A: These viruses contain a lipid enveloped and are of intermediate to large size. These viruses are the easiest group to inactivate since the lipid envelope is sensitive to many lipophilic compounds.

Category B: These viruses are the most difficult to inactivate. They include small non lipid containing viruses and those protected within a protein matrix (occlusion).

Category C: These viruses are intermediate in their facility of inactivation by chemical agents. They do not contain lipids but are usually larger than viruses in category B.

Viruses	Disinfection category
1. White spot disease	A
2. Taura syndrome	B
3. Yellow head disease – Yellow head virus	B
4. Infectious hypodermal and hematopoietic necrosis	B
5. Infectious myonecrosis	
6. MonodonBaculovirus (MBV)	B
7. Gill-associated virus	A
8. Monodon slow growth syndrome	?

The disinfection in bacteria can be divided in four groups depending on the cell wall nature.

Gram positive vegetative bacteria: Those tends to be most susceptible to disinfection.

Gram negative bacilli bacteria: Are most resistant to disinfectants agents than Gram negative cocci.

Mycobacteria: Tend to occupy an intermediate place between Gram-negative bacteria and bacterial spores.

Bacterial spores: Are most resistant to the action of disinfectants.

Reference table for water treatment and general disinfection

Chemical / disinfection method	Active ingredients	Dosage of active ingredient	Contact time	Scope	Application	Elimination of residues
Chlorine	Calcium hypochlorite	30ppm	1 hour	WSSV eradication in water	Water disinfection for hatcheries and grow-out	Sun light, aeration
		15ppm	1 hour plus 120 hours retention time	Zooplankton eradication	Water disinfection for hatcheries and grow-out	Sun light, aeration
		200ppm	1 hour	Surface disinfection	Disinfection of tanks and equipment	Dry out
		30ppm	1 min	Surface disinfection	Vehicles	Dry out, sun light
Formalin	Formaldehyde	100 ppm	30 sec	eggs, nauplii, PLs	Baths	Natural breakdown
Copper Sulfate	Copper	0.5ppm	48 hours	Zooplankton eradication (particularly rotifers)	Water disinfection for grow-out	Natural breakdown (10 days)
ADMA approved crusticide	Trichlorfon	0.5ppm	N/A	Crustacean eradication Zooplankton eradication except rotifers	Water disinfection for grow-out	Natural breakdown (10 days)
Quaternary Ammonium	Quaternary Ammonium	350ppm	<5 min	WSSV eradication and general disinfection	Foot bath, vehicle disinfection and disinfection of equipment	Natural breakdown
Potassium Permanganate	Potassium Permanganate	350ppm	<5 min	WSSV eradication and general disinfection	Foot bath, vehicle disinfection and disinfection of equipment	Natural breakdown
Povidine	Iodine	100 ppm	<5 min	WSSV eradication and	Hands dip and disinfection of delicate tools	Natural breakdown
		25 ppm	30 sec	eggs disinfection	Baths	
		50 ppm	30 sec	nauplii disinfection	Baths	
		200 ppm	1 min	foot dip	baths	
Ozone	Ozone	>0.5 mg/L for 10 minutes	8min for ORP values of 600-700	Eradication of any living organisms	Primary water treatment after mechanical filtration.	12 hours by oxidation (Bromine must be <0.05ppm)
UV	UV light	Radiation 200-300 nm	Irradiation must reach >10 mJ/cm2 in the incoming water flow	Eradication of micro-organisms bacteria and virus of category A	End of water treatment for hatcheries	N/A

SOP 18. Surveillance and prophylactic measures

Surveillance

1. Nucleus Breeding Center/ indoors broodstock tanks

1.1 Nucleus Breeding center

As per the ADMA operational surveillance protocol the following health monitoring must be done:

1.1.1 In case of Non-SPF broodstock, every batch of the spent females will be analyzed for WSSV and TSV by PCR. Samples of individual spent female will be pooled (ten samples for one pool) and analyzed for WSSV/TSV. In case of SPF broodstock, at least 10 samples per month will be analyzed for ADMA diseases list.

1.1.2 Daily mortality will be analyzed for WSSV and TSV by PCR. Pleopods from dead shrimp will be pooled (10 pleopods will be pooled in one sample).

1.1.3 If animals display any clinical signs they must be fixed for histological analysis.

1.1.4 PLs from SPF broodstock, while recommended, will not require further testing when moved within the same operation as long as the same level of biosecurity is maintained within the larval rearing process. PLs from SPF broodstock will require PCR certification for endemic pathogens when moved to a different operation from non-SPF broodstock will require going through cold challenge and PCR testing for WSSV and TSV.

1.1.5 150 Postlarvas will go through a cold challenge (See SOP 12. cold challenge). After the cold challenge, 3 pools of 50 PL each (total 150 PL per tank) will be analyzed for WSSV, IHHNV and TSV by PCR.

1.1.6 Each juvenile population will be sampled at least once per batch for WSSV, IHHNV and TSV by PCR. 3 subsamples of 50 pleopods each (total 150 shrimp) will be pooled in tube containing 95% ethanol.

1.2 Nauplii Production unit and indoor broodstock tanks/ponds

1.2.1 For non SPF stock

a) Daily mortality present in the NPU will be analyzed for WSSV and TSV by PCR. Pleopods from dead shrimp will be pooled (10 pleopods will be pooled in one sample).

b) Every batch of the spent females will be analyzed for WSSV and TSV by PCR. Every 10 shrimp pleopods will be pooled in 1 sample (unless broodstock are used more than once).

c) Any shrimp displaying any clinical signs will be fixed for histological analysis in the NPU every month (See SOP 14: Fixation of shrimp and crustacean samples in Davidson's fixative.

d) Broodstock in the NPU units will not be fed with any fresh feed imported from areas where WSSV, TSV and AHPND are endemic (See SOP 13 Fresh feed).

1.2.2 For SPF stock

a) Daily mortality present in the NPU will be analyzed for WSSV, IHHNV and TSV by PCR. Pleopods/gills from dead shrimp will be pooled (10 pleopods will be pooled in one sample).

b) Any shrimp displaying any clinical signs will be fixed for histological analysis in the NPU every month (See SOP15: Fixation of shrimp and crustacean samples in Davidson's fixative).

c) Broodstock in the NPU units will not be fed with any fresh feed imported from areas where WSSV, TSV and AHPND are endemic (See SOP 13 Fresh feed).

d) Only eggs and nauplii produced by broodstock without history of any of the ADMA listed diseases with emphasis in WSSV and TSV will be allowed to continue in production.

Eggs will be dipped into at least 25 ppm iodine for 30 minutes and then rinsed with seawater.
Nauplii will be dipped into at least 50 ppm iodine for 30 minutes and then rinsed with sea water.

2. Larval Rearing / Post larval Rearing / Raceways

As per ADMA operational surveillance protocol the following health monitoring

must be done:

2.1 Prior stocking the PLs, into the grow-out ponds / outdoors nursery ponds, each tank must be tested for WSSV and TSV by PCR. 150postlarvas will go through a cold challenge (See SOP 12: Cold challenge). After the cold challenge, 3 pools of 50 PL each (total 150 PL per tank) will be analyzed for WSSV and TSV by PCR. Only if negative results are obtained, the PLs could be stocked into the grow-ponds / outdoors nursery ponds (See SOP 14: Sampling for PCR analysis).

2.2 PLs from hatchery to indoors nursery will not required additional testing if the same biosecurity standard are met.

2.3 Prior stocking the juveniles into the grow-out ponds, each pond must be tested for WSSV and TSV by PCR. 150 juvenile will go through a cold challenge (See SOP 12: Cold challenge). After the cold challenge, 3 pools of 50 pleopods each (total 150 pleopods per tank) will be analyzed for WSSV and TSV by PCR. Only if negative results are obtained, the juveniles could be stocked into the grow-ponds. (See SOP 14: Sampling for PCR analysis).

2.4 Any unusual mortality will be analyzed by PCR for WSSV and TSV. For this, 10 PLs will be pooled in one sample.

3. Outdoor Broodstock ponds

Only broodstock stocks raise isolated from the farm areas will be allowed to be stock in outdoor facilities. ADMA will approve the facilities before use them as a broodstock ponds.

3.1 All broodstock must be cold challenged and PCR tested for endemic pathogens before moving to maturation.

3.2 Each broodstock pond will be sampled at least once per month for WSSV and TSV by PCR (See SOP 14: Sampling for PCR analysis). 5 subsamples of 30 pleopods each (total 150 shrimp) will be pooled in tube containing 95% ethanol.

3.3 Five shrimp displaying any clinical sign will be fixed for histological analysis (See SOP 15 fixation of shrimp and crustacean samples in Davidson's fixative) in a monthly basis.

3.4 It is recommended to take plankton samples from the broodstock ponds for WSSV and TSV (See SOP 16: Plankton sampling).

3.5 Any mortality observed in broodstock ponds will be analyzed. Samples of recent dead shrimp must be analyzed for WSSV and TSV by PCR in pools of 5 (five recent dead shrimp pleopods will conform 1 sample).

4. Outdoor Nursery and grow out ponds

4.1 Each nursery ponds will be sampled at least once per month for WSSV and TSV by PCR). 5 subsamples of 30 pleopods each (total 150 shrimp) will be pooled in tube containing 95% ethanol.

4.2 If any mortality is observed in nursery ponds, samples of recent dead or moribund shrimp must be analyzed for WSSV and TSV by PCR or field rapid test (e.g. shrimple)/ in pools of 5 (five recent dead shrimp pleopods = 1 sample) or field test system (e.g. shrimple, pockit etc). Five samples of shrimp displaying clinical signs will be fixed for histopathology.

4.3 The sampling for grow-out ponds will be conducted based on the presence of clinical signs (See SOP 7: Sampling a shrimp ponds for sanitary analysis). Each grow-out pond will be sampled at least once during the first month for WSSV by PCR. If history of TSV, samples for TSV will be taken as well. 3 subsamples of 50 pleopods each (total 150 shrimp) will be pooled in a tube containing 95% ethanol. The samples will be taken according the SOP 7 Criteria for shrimp sampling in ponds/tanks for sanitary analysis.

4.4 In case of any mortality or abnormal presence of birds observed in grow-out ponds, samples of moribund or recent dead shrimp must be analyzed for WSSV by PCR (5 recent dead shrimp pleopods = 1 pool sample) (See SOP 14: sampling for PCR analysis) or a field test system (e.g. shrimple, pockitetc).

4.5 In case of presence of moribund shrimp, samples of moribund shrimp (at least 5) must be fixed in Davidson fixative for histological analysis. See SOP 15 fixation of shrimp and crustacean samples in Davidson's fixative.

4.6 If there is a lower final survival in any grow-out pond (significantly lower than the final survival average), the cause of the mortality should be clearly explained and sanitary results must clearly stated that analysis for WSSV and TSV was conducted.

5. Wild population

5.1 It is recommended to analyze crustacean samples taken from different areas of the farm including main canal, feeder canals and any mangrove area near the farm. Samples of gills (preferred), pleopods or hemolymph will be taken from the crustacean present in each area and pooled (Each 5 crustaceans samples will conform=1 pool) for WSSV analysis. At least 1 pools sample per area per month will be analyzed by PCR.

5.2 Plankton samples must be analyzed by PCR for WSSV and TSV. Five samples of the main feeder canal per month must be preserved in 95% ethanol (See SOP 16 Plankton samples for PCR analysis).

ANEXURE 1 LIST OF THE REFERENCE LABS FOR SHRIMP DISEASES

Infectious hypodermal and haematopoietic necrosis	**DrJie Huang** Maricultural Organism Disease Control and Molecular Pathology Laboratory, Yellow Sea Fisheries Research Institute (YSFRI), Chinese Academy of Fishery Sciences #106 Nanjing Road, Qingdao, Shandong Province 266071, CHINA (PEOPLE'S REPUBLIC OF)) Tel.: (86-532) 85.82.30.62 ext. 802, Fax: (86-532) 85.81.15.14; E-mail: huangjie@ysfri.ac.cn; aqudis@public.qd.sd.cn Web site: www.ysfri.ac.cn **Prof. D. Lightner** Aquaculture Pathology Laboratory, Department of Veterinary Science and Microbiology, University of Arizona, 1117 E. Lowell, Building 90, Tucson AZ 85721, UNITED STATES OF AMERICA Tel.: (1-520) 621.84.14, Fax: (1-520) 621.48.99 E-mail: dvl@u.arizona.edu

Infectious myonecrosis	**Prof. D. Lightner** Aquaculture Pathology Laboratory, Department of Veterinary Science and Microbiology, University of Arizona, 1117 E. Lowell, Building 90, Tucson AZ 85721, UNITED STATES OF AMERICA Tel.: (1-520) 621.84.14, Fax: (1-520) 621.48.99 E-mail: dvl@u.arizona.edu
Necrotisinghepatopancreatitis	Awaiting receipt of application
Taura syndrome	**Prof. D. Lightner** Aquaculture Pathology Laboratory, Department of Veterinary Science and Microbiology, University of Arizona, 1117 E. Lowell, Building 90, Tucson AZ 85721, UNITED STATES OF

	AMERICA Tel.: (1-520) 621.84.14, Fax: (1-520) 621.48.99 E-mail: dvl@u.arizona.edu
White spot disease	**DrJie Huang** Maricultural Organism Disease Control and Molecular Pathology Laboratory, Yellow Sea Fisheries Research Institute (YSFRI), Chinese Academy of Fishery Sciences #106 Nanjing Road, Qingdao, Shandong Province 266071, CHINA (PEOPLE'S REPUBLIC OF)) Tel.: (86-532) 85.82.30.62 ext. 802, Fax: (86-532) 85.81.15.14; E-mail: huangjie@ysfri.ac.cn; aqudis@public.qd.sd.cn Web site: www.ysfri.ac.cn **Dr G. Lo** Department of Life Science, Institute of Zoology, National Taiwan University, 1 Roosevelt Road, Section 4, Taipei, CHINESE TAIPEI Tel.: (886-2) 23.63.35.62, Fax: (886-2) 23.63.81.79 E-mail: gracelow@ntu.edu.tw **Prof. D. Lightner** Aquaculture Pathology Laboratory, Department of Veterinary Science and Microbiology, University of Arizona, 1117 E. Lowell, Building 90, Tucson AZ 85721, UNITED STATES OF AMERICA Tel.: (1-520) 621.84.14, Fax: (1-520) 621.48.99 E-mail: dvl@u.arizona.edu
White tail disease	**Dr A. SaitSahul Hameed** Aquaculture Biotechnology Division, Department of Zoology, C. Abdul Hakeem College, Melvisharam-632 509, Vellore Dt. Tamil Nadu, INDIA Tel.: (91-4172) 269.487, Fax: (91-4172) 269.487 E-mail: cah_sahul@hotmail.com
Yellow head disease	**Dr P. Walker** Australia Animal Health Laboratory (AAHL), CSIRO Livestock Industries Private Bag 24, Geelong, VIC 3220, AUSTRALIA Tel.: (61-3) 52.27.54.65, Fax: (61-3) 52.27.55.55 E-mail: peter.walker@csiro.au

Spherical baculovirosis (*Penaeus*monodon-type baculovirus)	**Dr G. Lo** Department of Life Science, Institute of Zoology, National Taiwan University, 1 Roosevelt Road, Section 4, Taipei, CHINESE TAIPEI Tel.: (886-2) 23.63.35.62, Fax: (886-2) 23.63.81.79 E-mail: gracelow@ntu.edu.tw

Spherical baculovirosis (*Penaeusmonodon*-type baculovirus); Tetrahedral baculovirosis (*Baculoviruspenaei*)	**Prof. D. Lightner** Aquaculture Pathology Laboratory, Department of Veterinary Science and Microbiology, University of Arizona, 1117 E. Lowell, Building 90, Tucson AZ 85721, UNITED STATES OF AMERICA Tel.: (1-520) 621.84.14, Fax: (1-520) 621.48.99 E-mail: dvl@u.arizona.edu

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