THAI AGRICULTURAL STANDARD
TAS 10450-2007

DIAGNOSIS OF TAURA SYNDROME IN SHRIMP

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The Thai Agricultural Standard (TAS) on Diagnosis of Taura Syndrome in Shrimp establishes the provisions on certified free status of Taura syndrome in shrimp and the provisions will be used as a manual for laboratories using histopathological method and reverse transcriptase polymerase chain reaction (RT-PCR) method to support the production system and ensure the quality of shrimp and its products for domestic and export markets.

The standard is based on the information of the following documents:


Remark:
The standard title has been amended from “Thai Agricultural Commodity and Food Standard (TACFS)” to “Thai Agricultural Standard (TAS)” in accordance with the enforcement of The Agricultural Standards Act B.E. 2551 (2008).
NOTIFICATION OF THE NATIONAL COMMITTEE ON AGRICULTURAL COMMODITY AND FOOD STANDARDS

SUBJECT: THAI AGRICULTURAL COMMODITY AND FOOD STANDARD:
DIAGNOSIS OF TAURA SYNDROME IN SHRIMP
B.E.2550 (2007)

The resolution of the 1/2550 session of the National Committee on Agricultural Commodity and Food Standards dated on 2 May B.E.2550 (2007) endorsed the Thai Agricultural Commodity and Food Standard entitled Diagnosis of Taura Syndrome in Shrimp to improve the quality, facilitate trade and protect consumers’ health.

By virtue of the Cabinet Resolution on Appointment and Authorization of the National Committee on Agricultural Commodity and Food Standards dated 3 April B.E.2550 (2007), the notification on Thai Agricultural Commodity and Food Standard entitled Diagnosis of Taura Syndrome in Shrimp is hereby issued as a voluntary standard. The details of which are attached herewith.

Notified on 29 May B.E.2550 (2007)

Professor Thira Sutabutra
Minister of the Ministry of Agriculture and Cooperatives
Chairperson of the National Committee on Agricultural Commodity and Food Standards
THAI AGRICULTURAL STANDARD
DIAGNOSIS OF TAURA SYNDROME IN SHRIMP

1. SCOPE

The Thai Agricultural Standard establishes details for the diagnosis of Taura syndrome in the laboratory using the histopathological method and the reverse transcriptase polymerase chain reaction (RT-PCR) method.

2. DEFINITIONS

For the purpose of this standard:

2.1 **Shrimp** mean animals in the genus *Penaeus*.

2.2 **Taura syndrome (TS)** means a disease occurring in shrimp, especially white shrimp, caused by Taura syndrome virus (TSV), characterized by lesions that appear as indeterminate red blotches on the shrimp’s shell in the area of the tail and appendages, usually resulting in swift death. If the shrimp survive longer, in the later stages the lesions turn to black.

2.3 **Diagnosis** means test or inspection to analyse and determine the presence of a disease.

2.4 **Larva** means newly hatched larval shrimp that will undergo 3 stages of metamorphosis of nauplius, zoea and mysis within approximately 13 days.

2.5 **Post larva (PL)** means shrimp that have the same appendages as adult shrimp, are about 5 mm long, and will grow from mysis stage to juvenile stage in about 25 days or more. The convention is to designate post larva shrimp with the abbreviation “PL” followed by a number that means the number of days since they passed from mysis to post larva stage. For example, “PL21” means shrimp that have been at the post larva stage for 21 days.

2.6 **Juvenile** means shrimp that are 2-3 cm long and are the same as adults but have not yet reached reproductive maturity.

2.7 **Adult** means fully mature shrimp that can reproduce.

2.8 **Haemolymph** means liquid containing heme that circulates in the haemocoels or spaces in the body of an invertebrate animal, which plays the same role as the blood and lymph of a vertebrate animal.

2.9 **Carrier** means an animal that carries a disease-causative agent but does not appear to be diseased.

2.10 **Presumptive test** means a fast and convenient laboratory procedure to test for a disease, such as rapid staining tests, histopathological tests or immunological tests.

2.11 **Confirmation test** means a laboratory procedure to confirm the results of a diagnosis, which is accepted to be highly specific and sensitive.
3. DIAGNOSIS

After receiving the results of a diagnostic technique for Taura syndrome in the laboratory through the histopathological method, reverse transcriptase polymerase chain reaction (RT-PCR) method, *In-situ* hybridization, or ELISA, the veterinarian or expert should also consider the clinical signs of the shrimp, the history of epizootics and the pathogenesis (Appendix A) to ensure the effectiveness of disease treatment or prevention. Each diagnosis method has a different level of sensitivity and specificity (Appendix B).

The presumptive laboratory test to diagnose Taura syndrome recommended in this standard is the histopathological method. If the results are negative, the diagnosis shall be confirmed by using the RT-PCR test or the *In-situ* hybridization method, which are highly sensitive and specific. The choice of which diagnosis test to use depends on the testing objectives and on the type of sample. For instance, when certifying that broodstock shrimp are disease free before breeding or when certifying that post larva shrimp are disease free before stocking into the grow-out ponds or in case where a disease is suspected but the shrimp show no clinical sign, the RT-PCR test shall be used. In the case of shrimp that are being raised in grow-out ponds, the histopathological or alternative methods may be used.

3.1 SAMPLING

For the number of samples to be taken, refer to Table C1 (Appendix C).

3.2 Histopathological method

The principle of this procedure is to detect evidence of the disease in shrimp tissue by staining fixed tissue with hematoxylin and eosin (H&E) and observing it under a light microscope.

3.2.1 Collection and storage of samples

(1) Take live shrimp and immerse them in chilled Davidson’s fixative (Appendix D, item D.1.1) of a volume that is approximately ten times the volume of the shrimp as follows:

(1.1) If the shrimp are nauplius to PL20 stage they may be kept whole in Davidson’s fixative.
(1.2) If the shrimp are PL21 stage to ≤ 3 g they may be kept whole in Davidson’s fixative but an incision shall be made lengthwise along the carapace to allow the fixative to reach the hepatopancreas.
(1.3) If the shrimp are ≥ 3 g Davidson’s fixative shall be injected into the shrimp’s mouth, under the back of the carapace, into the hepatopancreas and the abdomen from the third to the last pair of walking legs (parareopods) as well as all over the dorsal and ventral portions of the cephalothorax, using from 1 to 10 ml of Davidson’s fixative per shrimp, depending on shrimp size. The shell shall be incised lengthwise from the sixth abdominal segment to the cephalothorax.
(1.4) If the shrimp are over 12 g, Davidson’s fixative should be injected thoroughly into the cephalothorax and the ventral side of the body from cephalothorax to tail, after which the shrimp shall be cut in half in cross section between the cephalothorax and abdomen.
The samples shall be immersed in Davidson’s fixative for 24 to 48 hours, depending on the size, then transferred to 70% ethanol to extend the storage life.

3.2.2 Procedure

Samples stored as in 3.2.1 above shall be treated as follows:

1. Dehydrate by immersing in 95% ethanol and 100% ethanol in sequence.
2. Clear ethanol with xylene.
3. Impregnate the samples with paraffin.
4. Embed the samples in paraffin.
5. Thinly section the paraffin-embedded samples and place on microscope slides.
6. Stain with H&E.

   6.1 Liquefy the paraffin in the samples by heating the slides to 60°C for 30 minutes, then immerse in xylene.
   6.2 Rehydrate the sample by immersing the slide in 100% ethanol and 95% ethanol in sequence.
   6.3 Rinse the slide in running water for 5 minutes.
   6.4 Immerse the slide in Mayer’s hematoxylin for 5-7 minutes.
   6.5 Rinse the slide in running water for 15-30 seconds.
   6.6 Immerse the slide in cosin for 30-60 seconds.
   6.7 Dehydrate the slide by immersing in 95% ethanol and 100% ethanol in sequence.
   6.8 Clear ethanol by immersing in xylene.
   6.9 Add 1 drop Permount and cover with a cover slip.
   6.10 Observe under a light microscope.

3.2.3 Interpretation

The indications of TSV differ with the stage of the disease:

3.2.3.1 Acute infection

Lesions of necrotic tissue may be observed in the epithelial tissue under the shell along the shrimp’s body (Figure E 3), appendages, gills, digestive tract and stomach (Figure E.4). Sometimes lesions are apparent in other related tissues such as structural muscle adjoining the epithelium, but rarely in the epithelium of the antennal gland. No inflammation-related infiltration of haemocytes is observed. Eosinophilic to pale basophilic cytoplasmic inclusions (red- to blue-staining round bodies) can be seen in the infected cells and the nucleus may be deteriorated as seen by dark spot (pyknotic nuclei) or fragmented (karyorrhectic nuclei), giving the cells a peppered or buckshot-riddled appearance (Figure E5).

3.2.3.2 Transition or recovery phase

In shrimp that are recovering from TSV, fewer lesions are seen in the epithelial tissue but greater infiltration and accumulation of haemocytes results in melanisation. The cuticle may be seen to be breaking off and bacterial accumulation, such as *Vibrio* spp., may be observed.

3.2.3.3 Chronic infection

No lesions are observed in the subcuticular epithelium but eosinophilic cytoplasmic inclusions (spheroids) may be observed in the lymphoid organ (Figure E.6).
3.3 Reverse transcriptase polymerase chain reaction (RT-PCR) method

The principle of this method is to detect TSV’s RNA by first transcribing RNA to DNA and then multiplying the DNA exponentially up to a detectable level. The following method is adapted from the Manual of Diagnostic Tests for Aquatic Animals by OIE\(^1\) (2003).

3.3.1 Collection and storage of samples

(1) Put live shrimp along with the water in which they were raised in a plastic bag or other suitable container. Samples from different ponds or tanks shall be kept separately. Clearly label the samples and transport them live to the laboratory.

(2) If it is not possible to take live samples, put the dead samples in a plastic bag, seal it securely and pack it on ice, then deliver it to the laboratory within 24 hours. If it is not possible to send the samples to the laboratory within such time, freeze the specimens whole with dry ice or in a freezer that is -20°C or lower and send them to the laboratory.

(3) If it is not possible to obtain live or frozen samples, keep the samples cool in chilled 90-95% ethanol with ethanol of about 10 times the volume of the sample, and test within 3 days. If it is necessary to store the specimen for a longer time, then it may be fixed in fixative (Appendix D, item D.1.2.). For juvenile stage shrimp or older, collect a sample of the gills or the pleopods. If the shrimp are smaller than 3 g, they shall be fixed whole but shall be tested within 7 days.

(4) If the sample to be tested is haemolymph it shall be taken from a haemocoel in the abdomen under the first pair of pleopods (the ventral sinus) or the third to last pair of periopods (the cardiac sinus) (Figure E2) or from the heart using a needle and syringe that is preloaded with citrate buffer (Appendix D, item D.1.3) of a volume equal to the volume of the sample. The sample shall be frozen and delivered to the laboratory within 8 hours or:

(4.1) Store the haemolymph sample in RNA fixer as follows:

(4.1.1) Suction out the haemolymph sample in citrate buffer and place in a microcentrifuge tube and centrifuge at 500 g for 10 minutes at 4°C.

(4.1.2) Remove the supernatant, be careful not to disturb the haemocytes at the bottom of the tube.

(4.1.3) Add an RNA fixer such as trizol or other RNA purification test to fill the bottom of the microcentrifuge tube and gently mix.

(4.1.4) Store the sample at -20°C to -80°C until testing time.

(4.2) Drop 30 µl haemolymph onto Isocode™ filter paper (or other filter paper with the same properties) and leave to dry. Haemolymph may be stored for 3 months this way.

\(^1\)The Office International des Epizooties (OIE) or World Organisation for Animal Health is an international organisation consisting of representatives of governments that was established by international agreement in January 1924 to coordinate efforts to control epizootics in livestock.
3.3.2 Procedure

3.3.2.1 Extraction of RNA

1. The sample size depends on the type of sample (the following steps shall be performed at a temperature under 4° C):
   1.1 For large (juvenile or adult) shrimp, take 25-75 mg shrimp tissue and add 150 µl trizol. Crush, then add 850 µl trizol for a final volume of 1,000 µl.
   1.2 For PL shrimp, take 300 shrimp and add 1,000 µl trizol. Crush, then take just 150 µl of the mixture and add 850 µl trizol for a final volume of 1,000 µl.
   1.3 If the sample is haemocytes, add 750 µl trizol and mix for 20 seconds.
2. Incubate the sample from (1) at 25°C for 5 minutes.
3. Centrifuge at 12,000 g at 4°C for 10 minutes; pipette up the supernatant and transfer it to a new microcentrifuge tube.
4. Add 200 µl chloroform and mix for 20 seconds.
5. Incubate at 25°C for 10 minutes.
6. Centrifuge at 12,000 g at 25°C for 10 minutes; pipette up the supernatant and transfer it to a new microcentrifuge tube.
7. Add 670 µl isopropanol and mix.
8. Incubate at 25°C for at least 10 minutes or incubate at -20°C over night or at -70°C for 1 hour.
9. Centrifuge at 12,000 g at 25°C for 10 minutes; pipette off the supernatant and discard.
10. Rinse the pellet with 0.5 ml of 70% ethanol for at least 30 minutes at 25°C.
11. Centrifuge at 12,000 g at 25°C for 10 minutes. Pipette off the supernatant and discard.
12. Leave at room temperature for 20 minutes or until the pellet is dry.
13. Add 150 µl EDTA for every 50mg of original sample tissue, or if the original sample was 250 µl haemocyte, add 75 µl EDTA. Incubate at 55°C for 15 minutes. Mix gently and use for the next step to create complementary DNA.

3.3.2.2 Construction of complementary DNA

1. Use RNA extracted from shrimp haemolymph or shrimp tissue with a concentration of 1-100 ng/ml as the template RNA.
2. Test sample using the specific primers 9195 and 9992 (Table 3) for a PCR product of 231 base pair (bp).
3. For every diagnosis, there shall be a negative control, positive control, and blank (non-template control).
4. Commercial RT-PCR kits may be used for every step.
5. For suitable condition of 50 µl RT-PCR cocktail for TSV in shrimp, the following components shall be used:
   - primer (0.46 µmol each)

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
<th>G:C ratio</th>
<th>Melting Temperature</th>
</tr>
</thead>
<tbody>
<tr>
<td>9195</td>
<td>5’-TCA-ATG-AGA-GCT-TGG-TCC-3’</td>
<td>50</td>
<td>63°C</td>
</tr>
<tr>
<td>9992</td>
<td>5’-AAG-TAG-ACA-GCC-GCG-CTT-3’</td>
<td>55</td>
<td>69°C</td>
</tr>
</tbody>
</table>

Table 3. Nucleotide sequence of primers 9195 and 9992
- dNTPs (300 µmol each)
- rTth DNA polymerase (2.5 U/50 µl)
- manganese acetate (2.5 mmol) in 5X EZ buffer (Appendix D, item D.2.1)

**Note:** For tests on *P. stylirostris*, the amount of rTth DNA polymerase shall be increased to 5.0 U/50 µl).

(6) Insert the sample into the PCR machine for the heating stage. If the microcentrifuge tubes used are not heat resistant, add 50 µl mineral oil on top of the sample to prevent evaporation.

(7) Set the program for 1 cycle at 60° C for 30 minutes for the reverse transcription of template RNA to produce cDNA. Then increase the temperature to 94° C for 2 minutes.

**Note:** The above conditions are suitable for the automatic DNA thermal cycler 480 (Perkin Elmer Cetus, Norwalk, CT). For other models of thermal cyclers, the optimized conditions shall be determined using the positive control first.

### 3.3.2.3 Multiplying target DNA

(1) When the reverse transcription step is complete, multiply the DNA by setting the machine for 35 cycles at 94° C for 45 seconds for denaturing; 60° C for 45 seconds for annealing and extension at the same time; and finally the last cycle at 60° C for 7 minutes. Store the PCR products containing DNA at 4° C.

(2) Pipette the DNA solution out from under the mineral oil and deposit in a 0.5 ml microcentrifuge tube.

(3) Separate the DNA using agarose gel electrophoresis as follows:

(3.1) Add dye to the DNA solution and place 10 µl in the depressions containing 2% agarose gel.
(3.2) Apply electric current to separate the DNA in 0.5X TBE solution (Appendix D, item D.2.2).
(3.3) Stain with ethidium bromide (0.5 µg/ml) or other dye.
(3.4) Use a 100-bp DNA ladder as the marker.

**Note:** Other primers, time intervals, temperatures, chemicals or tools may be used for diagnosis if they are proven to be at least as sensitive and specific as this one and have been published in an academic journal.

### Precautions:

- To prevent contamination of DNA and RNase, the materials, equipment and work space shall be kept separately when extracting and mixing the PCR cocktail.
- Filter tips shall be used for mixing the PCR cocktail and preparing the template RNA.

### 3.3.3 Interpretation of results

(1) A 231 bp DNA strip means a positive reading compared to the positive control.
(2) No 231 bp DNA strip means a negative reading compared to the negative control.
3.4 *In-situ* hybridization with non-radioactive cDNA

The principle of this method is to detect TSV’s RNA or DNA in the shrimp tissue by using a cDNA strand to bind to the target RNA or DNA.

For this diagnosis method, refer to the Manual of Diagnostic Tests for Aquatic Animals by OIE (2003) to create one’s own DIG-labelled cDNA probe in the laboratory, or use a commercially prepared probe such as ShrimProbe™ kit, or a probe produced using the PCR method with primers 9155 and 9992.

3.4.1 Collection and storage of samples

As in the histopathological method (3.2.1), but after soaking in Davidson’s fixative for 24 hours, proceed directly to the next step. If it is not possible to proceed to the next step immediately, then use RNA friendly fixative (R-F fixative) (Appendix D, item D.3.3) and soak for 24-48 hours, then the samples may be stored in 70% ethanol for up to 2 weeks.

3.4.2 Procedure

3.4.2.1 Take the tissue samples that have been prepared in the same way as for the histopathological method in step 3.2.2 (5) to undergo a reaction with the DIG-labelled cDNA probe. From the paraffin-embedded samples, select the cuticular epithelium tissue or lymphoid organ tissue, or other tissues such as gill tissue or digestive tract tissue from shrimp in the acute or chronic stages of the disease. Section to a thickness of 4-6 µm and place on pre-treated slides or positively charged slides, then incubate at 60° C to partially melt the paraffin and proceed as follows:

3.4.2.2 Deparaffinisation
Immerse tissue in xylene twice, 5 minutes each time.

3.4.2.3 Rehydration
(1) Immerse tissue in 100% ethanol twice, 5 minutes each time.
(2) Immerse tissue in 95% ethanol twice, 5 minutes each time.
(3) Immerse tissue in 70% ethanol twice, 5 minutes each time.
(4) Immerse tissue in 50% ethanol twice, 5 minutes each time.
(5) Immerse tissue in distilled water for 5 minutes.

3.4.2.4 Pre-hybridisation treatment
(1) Drop proteinase K 10 µg/ml to completely cover the tissue and incubate at 37° C for 15 minutes; however, the most suitable concentration of proteinase K and the most suitable length of time varies, so test first for suitable parameters.
(2) Bathe the tissue in phosphate-buffer saline (PBS) (phosphate buffer 10 mmol, NaCl 150 mmol, pH 7.2) three times for 5 minutes each time.
(3) Drop acetic acid 20% to completely cover the tissue and leave for 20 seconds.
(4) Immerse in standard saline citrate (SSC) (NaCl 0.3 mol, sodium citrate 30 mmol, pH 7.0) 2X twice for 2 minutes each time.
(5) Immerse in distilled water for 5 minutes.
(6) Leave to dry thoroughly at room temperature.

3.4.2.5 Hybridisation

(1) Dissolve the DIG-labelled cDNA probe with hybridization buffer (Appendix D, item D.3.2) at the ratio of 1:20; combine; boil at 90-100° C for 5 minutes then immediately plunge the probe into ice for 5 minutes.

(2) Place 50 µl of the hybridization buffer with probe from (1) onto a cover slip; gently place the slide with the tissue sample on it down until it is in contact with the buffer; wait for the buffer to spread throughout the tissue, then incubate at 37° C in a moisture retention chamber over night.

3.4.2.6 Washing and blocking

(1) Remove the cover slip and immerse the sample tissue in SSC 2X at room temperature for 5 minutes.
(2) Immerse in SSC 2X at 37° C for 5 minutes.
(3) Immerse in SSC 1X at 37° C for 5 minutes.
(4) Immerse in SSC 0.5X at 37° C for 1 hour.
(5) Immerse in tris-buffered saline (TBS) (tris-HCl 50 mmol, NaCl 100 mmol, pH 7.2) for 5 minutes.
(6) Add one drop 1% skim milk to completely cover the tissue and leave at room temperature for 1 hour.
(7) Immerse in TBS pH 7.2 for 5 minutes.

3.4.2.7 Detection

(1) Dilute goat anti-digoxigenin in bovine serum albumin (BSA) 3% to a concentration of 1:300, drop to cover the tissue; leave at room temperature for 1 hour.
(2) Immerse the tissue in TBS pH 7.2 three times for 5 minutes each time.
(3) Immerse in TBS pH 9.5 for 5 minutes.
(4) Add one drop nitroblue tetrazolium (NBT)/5-bromo-4-chloro-3-indolyl phosphate (BCIP) to completely cover the tissue; leave at room temperature for 15-30 minutes.
(5) Immerse in distilled water for 3 minutes.
(6) Add a drop of nuclear fast red or Bismark brown dye to completely cover the tissue for 5 minutes.
(7) Immerse tissue in 50% ethanol twice, 5 minutes each time.
(8) Immerse tissue in 70% ethanol twice, 5 minutes each time.
(9) Immerse tissue in 95% ethanol twice, 5 minutes each time.
(10) Immerse tissue in 100% ethanol twice, 5 minutes each time.
(11) Immerse tissue in xylene twice, for 5 minutes each time.
(12) Cover with a cover slip and examine under a light microscope.

3.4.3 Interpretation

The cytoplasm of TSV-infected cells will stain blue to dark blue but the stain will not react with the karyorrhectic nuclear fragments and pyknotic nuclei.
APPENDIX A

EPIZOOTIOLOGY, PATHOGENESIS AND CLINICAL SIGNS OF TAURO SYNDROME

A1. EPIZOOTIOLOGY

Taura syndrome, or TS, is a serious epizootic in Pacific white shrimp (*Penaeus vannamei* or *Litopenaeus vannamei*) that is caused by Taura syndrome virus (TSV), which is a single-strand RNA (ssRNA) virus in the family Picornaviridae that lives in the cytoplasm of host cells. It is an icosahedronical non-enveloped virus with diameter 32 nm and density of 1.338 g/ml.

TS was first reported in 1992 at a Pacific white shrimp farm in Ecuador. After that the disease quickly spread in the American continent along the Pacific coast from Peru to Mexico, to Hawaii, and along the Atlantic coast in the Caribbean Sea and the Gulf of Mexico. In 2002 TS was reported in Asia after it was introduced with Pacific white shrimp imported from Central and South America. In 2003 an outbreak was first reported in Thailand on a shrimp farm in the central region. In 2004 it was detected in black tiger shrimp. TS has also been reported in *P. stylirostris, P. setiferus, P. schmitti, P. merguiensis* and *P. monodon*.

Because TS is a serious shrimp disease that has spread quickly, the Office International des Epizooties (OIE) added it to its list of aquatic animal diseases and the Thai government added it to a 2005 ministerial regulation under the 1956 Animal Epizootic Act.

A2. PATHOGENESIS

TSV invades the subcuticular epithelium of the cephalothorax, abdomen appendages, gills and digestive tract, resulting in lesions of necrotic tissue. Sometimes TS is found in related subcuticular tissues and striated muscle tissue but it is almost never detected in the antennal gland epithelial tissue. Also, no host-inflammatory response of haemocyte invasion is associated with TS. Shrimp that are infected with TSV may or may not display clinical signs, but most of those that die are larvae to juvenile shrimp. For shrimp that have reached the PL to sub-adult stages, TSV infection results in a mortality rate of 40-90%.

A3. CLINICAL SIGNS

Three phases have been identified:

A.3.1 Acute phase

The shrimp’s shell is red (Figure E.1a), especially around the tail and appendages (pleopods). The shrimp’s tail is redder than normal but the edges are pale (Figure E.1b). If the appendages are viewed under a light microscope, lesions of dead tissue can be observed. If the infected shrimp are moulting, they will have soft shells and the mortality rate will be high.

A.3.2 Transitional phase

Dark spots of melanisation of indeterminate shape can be seen on the gills (Figure E.1c). Shrimp observed at this stage might not have scattered chromatophores, their shells might not be soft and they might eat normally and have normal behavior.
A.3.3 Chronic phase

If the shrimp moult and do not die they will enter the chronic phase and no melanisation will be visible on the shells. In this phase the TSV is in the lymphoid organ spheroids (LOS) and the shrimp are carriers.
## APPENDIX B

### COMPARISON OF ACCURACY OF DIAGNOSIS TECHNIQUES

Table B.1 Comparison of the accuracy of different methods to diagnose Taura Syndrome

<table>
<thead>
<tr>
<th>Diagnosis Method</th>
<th>Screening</th>
<th>Presumptive</th>
<th>Confirmation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>larva</td>
<td>PL</td>
<td>juvenile</td>
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<tr>
<td>Gross signs</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Direct BF/LM microscope</td>
<td>-</td>
<td>-</td>
<td>-</td>
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<tr>
<td>Histopathology</td>
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<td>TEM</td>
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<tr>
<td>ELISA</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>DNA probes-\textit{in situ}</td>
<td>-</td>
<td>+++</td>
<td>+++</td>
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<tr>
<td>RT-PCR</td>
<td>-</td>
<td>+++</td>
<td>+++</td>
</tr>
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</table>

1 Adapted from the Manual of Diagnostic Tests for Aquatic Animals (OIE, 2003).

- = no test available or unsuitable  
+ = for limited use  
++ = standard test, commonly used, with good efficiency and specificity  
+++ = recommended test with high specificity and high sensitivity

BF/LM = bright field/light microscope
### APPENDIX C

**SAMPLING PLAN**

**Table C1. Sampling guide**

<table>
<thead>
<tr>
<th>Lot sizes (number of shrimp)</th>
<th>Number of samples per estimated disease prevalence</th>
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<tr>
<td></td>
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1 Adapted from the Manual of Diagnostic Tests for Aquatic Animals (OIE, 2003).

**Note:** The sample size depends on the estimated prevalence of the disease and the lot size, or the total number of shrimp in the pond.

1) For PL shrimp the sample size shall be based on the estimated disease prevalence of 1% or 2%. Live shrimp shall be taken from 5 different spots around the pond. For testing broodstock shrimp, all of the shrimp shall be tested or the number of samples shall be based on the estimated prevalence of 5%. For juvenile shrimp, the estimated prevalence may be 2-10%.

2) Interpretation: for example, if the estimate prevalence is 2%,
   - Positive diagnosis means 2% or more of the samples are infected.
   - Negative diagnosis means less than 2% of the samples are infected or none of them are infected.
APPENDIX D

CHEMICAL SOLUTION PREPARATION

D.1 SOLUTION FOR PREPARING SAMPLES

D.1.1 Davidson’s fixative: 1,000 ml

- Absolute ethanol: 330 ml
- 40% formalin: 220 ml
- Glacial acetic acid: 115 ml
- Distilled water: 335 ml

D.1.2 Fixative: 1,000 ml

- Absolute ethanol: 800 ml
- Glycerol: 200 ml
- β-mercaptoethanol: 2.5 ml

D.1.3 Haemolymph fixative: 1,000 ml

- NaCL: 26.3 g
- Trisodium citrate: 8.8 g
- Citric acid: 5.5 g
- EDTA: 3.7 g

- Add 700 ml water then adjust to pH 7.0 and autoclave.
- Cool to room temperature; add 100 ml 1 mol glucose (36 g glucose dissolved in 200 ml distilled water).
- Add water to 1,000 ml.

D.2 SOLUTION FOR RT-PCR

D.2.1 5X EZ buffer, pH 8.2

- 25 mmol bicine
- 57.5 mmol potassium acetate
- 40% (w/v) glycerol

D.2.2 5X TBE (prepare 1,000 ml)

- tris base: 54 g
- boric acid: 27.5 g
- 0.5 mol EDTA (pH 8): 20 ml

D.3 EQUIPMENT AND SOLUTIONS FOR IN SITU HYBRIDISATION

D.3.1 Pre-treated slides

1. Wash slides in 1 mol HCl for 20 minutes, then rinse in tap water for 30 minutes.
2. Dip in distilled water then leave to dry.
3. Dip in 2% organosilane (gamma-aminopropyltriethoxy silane) in acetone; leave at air temperature for 30 minutes.
4. Rinse in tap water for 30 minutes, then dip in distilled water 3 times, changing the water each time.
5. Incubate at 100° C for 1 hour.
(6) Store at room temperature in a dust-free place.

D.3.2 Hybridisation buffer
- Deionized formamide: 1 ml
- Dextran sulphate: 0.2 g
- 20X SSPE: 0.6 ml
- Yeast tRNA (10 mg/ml): 0.2 ml

D.3.3 RNA friendly fixative (R-F fixative): 1,000 ml
- 100% formalin (37-39% formaldehyde): 349 ml
- 95% ethanol: 407 ml
- Distilled water: 222 ml
- Ammonium hydroxide (28-30% as NH₃): 22 ml
Figure E.1 Acute-phase signs of Taura syndrome in Pacific white shrimp. The shrimp body is reddish and the shell is soft (a) the tail is redder than usual but the edges are pale (b) If the shrimp survive the transitional phase of the disease, black melanisation can be seen on the shell (c) (by courtesy of Dr. Jiraporn Kasornchandra).
Figure E. 2 Method for collecting haemolymph from the ventral sinus or the cardiac sinus (arrow). (by courtesy of Assoc. Prof. Dr. Jennuch Wongtawatchai, DVM).

Figure E.3 Histopathological characteristics of Taura syndrome: TSV lesions are observable in the subcuticular epithelium (H&E stain); magnified 1,000X. (by courtesy of Dr. Timothy W. Flegel).

Figure E.4 Histopathological characteristics of Taura syndrome: lesions of necrotic tissue are observable in the digestive tract epithelium (H&E stain); magnified 400X. (by courtesy of Assoc. Prof. Dr. Jennuch Wongtawatchai, DVM).
**Figure E.5** Histopathological characteristics of Taura syndrome: eosinophilic to pale basophilic cytoplasmic inclusions and pyknotic nuclei can be observed in the cytoplasm of infected cells (H&E stain); magnified 1,000X. (by courtesy of Dr. Timothy W. Flegel).

**Figure E.6** Histopathological characteristics of Taura syndrome: eosinophilic cytoplasmic inclusions are apparent in the lymphoid organ (pyknotic and karyorrhectic nuclei) (H&E stain); magnified 1,000X (a, b). (by courtesy of Dr. Jiraporn Kasornchandra).
APPENDIX F

UNITS

The units and their abbreviations used in this standard follow the International System of Units (*Le Système International d’Unités*):

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<th>Unit</th>
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<tr>
<td></td>
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