The study of virus collation with the polymerase chain reaction (PCR) method in export fishery commodities

L Kurniawati¹ and K T Pursetyo²,³*

¹Program Study of Aquaculture, Faculty of Fisheries and Marine, Universitas Airlangga, Kampus C Jalan Mulyorejo, Surabaya 60115, East Java, Indonesia
²Department of Marine, Faculty of Fisheries and Marine, Universitas Airlangga, Kampus C Jalan Mulyorejo, Surabaya 60115, East Java, Indonesia
³Faculty of Fisheries and Food Science, Universiti Malaysia Terengganu, 21030 Kuala Nerus, Terengganu, Malaysia

*Corresponding author: kustiawan@fpk.unair.ac.id

Abstract. Fish trade traffic from within and outside the country leads to the development of changes in pests and fish diseases in Indonesia. One of the infectious diseases known to attack the Indonesian exports of fishery commodities is a virus and can be identified using Polymerase Chain Reaction (PCR). The goal is to know the types of fish and shrimp and their export commodities with the examined samples virus and virus checking procedures with PCR. The method was used descriptive method. Stages of PCR consist of extraction of DNA/RNA, amplification using the kit IQ2000™ and Access Quick RT-PCR System, electrophoresis, and diagnostic test results on Uvidoc. Types of fish and shrimp samples for examination were freshwater fish, lobster, broodstock vanamei, red lobster, fries, and seawater ornamental shrimp—the type of virus that would be examined in accordance with the provision of export destinations. During street vendors’ activities, total samples of the fish and shrimp virus examination of export commodities were 39 samples declared negative, whereas two samples declared positive were WSSV.

1. Introduction

The increase in the value of Indonesia’s export fishery commodities in the productive market from 2010 to 2011 was 9.5% [1]. This increase is due to Indonesia having water fertility based on the food chain [2, 3]. Vital components of the food chain in fresh and marine waters are phytoplankton and zooplankton [4, 5]. The plankton community describes water quality because plankton cannot isolate itself from waters such as shellfish, which can close their shells when conditions are unfavorable [6, 7]. Plankton accumulates the effects of water quality changes that explain plankton and its interactions with the environment to manage water quality [8, 9]. Phytoplankton responds to changes in light, nutrients, and sediment and responds to eating by zooplankton [10,11]. The abundance and types of phytoplankton in the waters can provide information about whether or not the condition of water quality affects water quality management [12,13]. For example, we must know the phytoplankton species, such as fish, shellfish, and humans. However, small amounts of toxic and dangerous phytoplankton can cause a phytoplankton population explosion (blooming) due to increased in nutrient concentrations in waters [14,15].

The fishery commodities that Indonesia mostly exported were shrimp, tuna, and other fish to several countries such as Japan, the United States, the European Union, and other countries. The
existence of fish trade traffic from within and outside the country has led to the development of pests and fish diseases in Indonesia [16].

According to Afrianto and Liviawaty [17], the fish disease is anything that can cause disturbance to fish, either directly or indirectly. Based on the type, the term disease in fish is divided into two, namely infectious and non-infectious diseases. Infectious disease can be defined as a disease in fish caused by microorganisms, such as viruses, bacteria, fungi, and parasites. In contrast, non-infectious diseases are diseases in fish caused by non-pathogenic factors as nutrition, water quality, toxicity, and genetics.

One of the infectious diseases known to attack export fishery commodities in Indonesia is that of a virus. The types of fish and shrimp viruses that are well known in Indonesia are a yellow head virus, which causes yellow head disease in shrimp, white spot syndrome virus, which causes white spot disease in shrimp, and koi herpes virus, which causes koi herpes. Virus disease in the Cyprinidae group and other viral infections [16].

Identifying fish disease-causing organisms is carried out at the level of parasites, mycotic, bacteria, and viruses using conventional methods and Polymerase Chain Reaction (PCR) [18]. Polymerase Chain Reaction (PCR) is an enzymatic method to multiply a particular nucleotide sequence exponentially in vitro [19]. One of the uses of the Polymerase Chain Reaction (PCR) method in fisheries is to determine the condition of the nucleotide sequence of a DNA so that viruses that attack fishery commodities can be detected. Based on this background, a study on virus examination in fish and shrimp for export commodities was carried out using the Polymerase Chain Reaction (PCR) method at the Molecular Biology Laboratory.

2. Materials and methods

2.1. Tools and Materials

The tools used in virus examination using the PCR method include (1) Lab Coat, (2) Latex Glove, (3) Mortal, (4) Thermoblock, (5) Vortex, (6) Microtypes, (7) Thermocycler brand Biometra, (8) Biorad Power-Pac Electrophoresis Unit, (9) Monitor, (10) Printer, (11) Uvidoc, (12) Microtube, (13) Office Stationery, (14) Spinner, (15) Centrifuge, (16) Stopwatch, (17) Paste, (18) Freezer, (19) Refrigerator/refrigerator, (20) Sectio device, (21) Eppendorf tube, (22) Eppendorf tube and (23) Micropipette.

Materials used in the DNA virus examination process include: (1) 70% ethanol, (2) dodecyl trimethylammonium bromide (DTAB) solution, (3) 95% ethanol, (4) cetyltrimethylammonium bromide (CTAB) solution, (5) chloroform, (6) Dissolve Solution, (7) Lysis Buffer Solution, (8) Quick RT-PCR System Access Kit Reagent, (9) Tris-EDTA (TE) Buffer, (10) Reagent IQ 2000 kit produced by Farming IntelliGene Tech. Corp. Taiwan, (11) agarose, (12) RNA Extraction Solution, (13) diethylpyrocarbonate (DEPC) ddH2O, (14) Isopropanol, (15) Nuclease Free Water, (16) aquades, (17) 75% alcohol, (18) loading-dye, (19) DNA marker and (20) Ethidium Bromide (EtBr).

2.2. Method of conventional PCR

The virus examination activity at the Soekarno-Hatta BBKIPM-KHP was carried out at the Molecular Biology Laboratory. This activity is carried out if there is a sample examination request submitted by the applicant (sample owner). Export fishery commodities that are included in fishery commodities that will be trafficked abroad are also sampled at BBKIPM-KHP Soekarno-Hatta, both through export activities (E) and sampling (PS). The inspection of one sample takes 1-2 days at most after the sample is submitted.

During the study activity, 39 samples of exported fish and shrimp species were tested for viruses. The sample consisted of 27 samples of fish species and 12 samples of shrimp species. The type of virus to be checked is based on the provisions of the export destination country. Virus examination at the Soekarno-Hatta BBKIPM-KHP was carried out using the conventional PCR method. The kit used for virus testing is the IQ2000™ detection kit manufactured by Farming IntelliGene Tech. Corp. Taiwan and the Access Quick RT-PCR System. In examining viruses, several things must be prepared.
in advance, including preparing tools and materials, sterilization of tools, and sample necropsy. The virus examination stage itself consists of four processes, namely: nucleic acid extraction, amplification, electrophoresis,

2.3. Sterilization Tool
Sterilization is an effective process to kill or remove infectious agents or contaminants (such as fungi, bacteria, and viruses) from surfaces, equipment, food, drugs, or biological culture media [20]. The presence of contaminants or infectious agents can affect the results of virus examination using the PCR method so that the equipment to be used must be sterilized first.

The process of sterilizing the equipment used in the Soekarno-Hatta BBKIPM-KHP laboratory uses two methods, namely, by using UV light and autoclave. The sterilization process is included in the physical sterilization method, namely using heating with steam (autoclave) and radiation (UV light) [20].

Sterilization using UV light is used for every laboratory room, aiming to sterilize large rooms and equipment. UV light in the laboratory is turned on when all the hall workers are done carrying out their work in the laboratory. The autoclave sterilization process is used to sterilize single-use equipment such as microtubes and Eppendorf tubes. The microtube used was 0.5 ml in size for the amplification stage, and the 1.5 ml Eppendorf tube was used for the extraction stage. The sterilization process is carried out in the preparation and sterilization room. The microtip that will be sterilized beforehand is placed in its place. The microtip arrangement was adjusted to the size, namely 0.5-10 µl, 20-100 µl, and 200-1000 µl microtypes.

Microtube and the Eppendorf tube was placed in a glass Beaker and wrapped in paper. The microtubes, Eppendorf tubes, and micro types wrapped in the paper were sterilized by autoclave at a temperature of 121ºC, 1 atm for 15 minutes. After sterilization, the microtubes and macro types are stored in a unique cabinet to store microtubes, Eppendorf tubes, and micro types.

2.4. Sample Necropsy
The samples that have been recorded are surgically removed to remove target organs suspected of being infected with the virus. This process is known as necropsy. The necropsy activity is carried out in the preparation room, which is also the Parasite Laboratory room. Sampling for the necropsy process was carried out randomly. The target organs for virus examination in fry or seed are all members of the body. First, the fry is removed from the plastic, then filtered and taken approximately 30 grams, then put into the specimen container labeled the sample code containing 70% ethanol. The function of 70% ethanol is to preserve samples to extend shelf life [21].

Large samples such as koi, parent vanamei, Cobia, and others, the target organs were taken in the form of muscles, brain, gills, and eyes. The target organ is inserted into a labeled specimen container containing 70% ethanol. The sample placed in a tube containing ethanol can be directly extracted or stored in the refrigerator. BBKIPM-KHP Soekarno-Hatta also received a comparative test with other centers or laboratories in the form of live samples, target organs that had been given ethanol, or the results of sample extraction.

2.5. Nucleic Acid Extraction
The nucleic acid extraction process is the most important part of the virus examination process because the extraction results will affect the amount and quality of nucleic acids produced so that later it will affect the further analysis process. This is in accordance with the statement by Surfianti et al. [22] that extraction is the initial stage in the DNA/RNA separation process and is an important stage for the next test stage, so it must be free from contamination. During the study activities, the DNA extraction process used at BBKIPM-KHP Soekarno-Hatta consisted of two methods: the lysis buffer method and the cetyltrimethylammonium bromide (CTAB) - dodecyl trimethylammonium bromide (DTAB) method. The RNA extraction process was carried out using the RNA Extraction method.
2.6. Virus Amplification
The extracted nucleic acid sample is then used in the amplification process. Amplification is a process to multiply target DNA or increase the number of existing target DNA [23]. The amplification process of viral DNA at BBKIPM-KHP Soekarno-Hatta was carried out in the amplification room using a conventional PCR (thermalcycler) machine. This is in accordance with Pranawaty et al. [23] statement that DNA amplification is carried out with the help of a thermalcycler or PCR machine. The reagents used in this process are the IQ2000 ™ kit and the Access Quick RT-PCR System. The IQ2000 ™ kit reagent is used for the amplification of DNA viruses, while for RNA it uses the IQ2000 ™ kit reagent and the Access Quick RT-PCR System.

The amplification process of RNA viruses is different from DNA, because RNA as a template cannot be carried out in the PCR process. The process of RNA amplification in PCR is known as reverse transcription-polymerase chain reaction (RT-PCR) because, in the RNA amplification process, a reverse transcriptase enzyme (Reverse Transcription) must be added which functions to reverse transcribe the mRNA molecules in the sample (complementary DNA) [24]. In the RNA amplification process, the reverse transcriptase enzyme (Reverse Transcription) must be added in the RT Enzyme Mix (if using the IQ2000 reagent) or AMV Access Quick (if using the Access Quick RT-PCR System reagent).

3. Result and discussion
The type of virus to be checked for export fishery commodities at BBKIPM-KHP Soekarno-Hatta is adjusted to the export destination countries' provisions in the Draft Guidelines for Examination of Certain Fish Diseases or Quarantined Fish Diseases in Fishery Products for Conditional Countries in 2011 [25]. The draft contains the types of fish diseases included in the OIE notification list in 2010 and standard methods for diagnosing fish pests and diseases based on the provisions of the export destination countries included in the 2011 OIE notification. Technical requirements for the export of fishery products based on destination countries are also contained in the draft. Therefore, the exporting country must ensure that the fish shipped come from the disease-free areas required by the destination country.

Countries that are export destinations for fishery commodities at BBKIPM-KHP Soekarno-Hatta include Turkey, South Korea, Hong Kong, China, Japan, Vietnam, Singapore, Malaysia, European Union, Australia, Spain, Germany, England, etc. These countries have required the types of fish pests that must be checked before being shipped [25].

To ensure that export fishery commodities to be sent are disease-free as required by the export destination country, it is necessary to check pests and fish diseases. Viruses are one of the pests and diseases of fish needed for inspection by the state condition. One of the methods used in examining viral pests and fish diseases at BBKIPM-KHP Soekarno-Hatta, which is also the standard method of diagnosing fish pests and diseases based on the 2011 OIE list, is the Polymerase Chain Reaction (PCR) method.

PS sample. 019 and PS. 020, the PCR test results tested positive for the WSSV virus. The two samples’ export destination countries are the European Union and Singapore, a list of countries requiring WSSV testing before shipping fishery commodities to be shipped [25]. Also, in the table above, other samples are sent to export destination countries, requiring viral diseases to be tested. For example, the E. 1291 sample, which will be sent to Turkey involves testing for the Spring Viremia Carp (SVC) virus and Epizootic Hematopoetic Necrosis Virus (EHNV) before delivery of these fishery commodities [25].

After testing, the examination results will be displayed in the Analysis Result Sheet and Test Result Report (LHP). Based on the LHP, which will be given to the sample owner as evidence of the examination results that have been carried out. The Health Certificate can be used as legal evidence stating that the fishery commodity to be trafficked is not infected with quarantine fish pests and diseases or certain fish pests required by the destination country. For samples whose test results are
positive, the health certificate cannot be issued yet and will be re-tested by three different analysts for the tested virus.

During the study activity, from 41 samples examined from 14 January 2013 to 14 February 2013, two samples tested positive for the WSSV virus. The two samples are PS samples. 019 and PS. 020, where both of them were healthy fish when checked. That may be the samples tested positive for the WSSV virus were healthy fish samples with carrier status as carriers of the WSSV virus. Thirty-nine other samples tested negative for the virus. BBKIPM-KHP Soekarno-Hatta can issue the sample a Health Certificate as proof that the fishery commodities to be trafficked are not infected with quarantine fish pests and diseases or certain fish pests required by the destination country.

4. Conclusion

Virus inspection activities on export fishery commodities at BBKIPM-KHP Soekarno-Hatta began with receiving samples, collecting sample data, necropsy, and testing with the Polymerase Chain Reaction (PCR) method. During the study activity, the samples that tested negative for the export fishery commodity virus were 39 samples, while the samples that were stated positive WSSV amounted to two samples.

5. References

[1] Marine and Fisheries Ministry 2011 Marine and Fisheries in Figures 2011 Jakarta 33-35.
[2] Azmi K A, Arsad S, Sari L A 2020 IOP Conference Series: Earth and Environmental Science 441(1).
[3] Sari L A, Pursetyo K T, Arsad S, Masithah E D, Setiawan E, and Affandi M 2019 Pollut. Res. 38 S27-S32.
[4] Syaifudin M, Sulmartiwi L, and Andriyono S 2017 JAFH. 6(1) 41-47.
[5] Pratama N A, Rahardja B S and Sari L A 2020 IOP Conference Series: Earth and Environmental Science 441(1).
[6] Putri A D A and Tjahjaningsih W 2018 JAFH. 7(3) 111-117.
[7] Sari L A, Wulansari P D, Nindarwi D D, Arsad S, and Affandi M 2019 EEC 25 S26-S31.
[8] Sari L A, Satyantini W H, Manan A, Pursetyo K T, and Dewi N N 2018 IOP Conference Series: Earth and Environmental Science 137(1).
[9] Sari L A, Masithah E D, and Alamsjah M A. 2018 JFMR. 2(1) 9-14.
[10] Rinawati M, Sari L A, and Pursetyo K T 2020 IOP Conference Series: Earth and Environmental Science 441(1).
[11] Damayanti K Y, Mubarak A S, and Sari L A 2020 IOP Conference Series: Earth and Environmental Science 441(1).
[12] Liyana S H, Sari L A, Dewi N N, Masithah E D, Sahidu A M, and Pursetyo K T 2019 IOP Conference Series: Earth and Environmental Science 236(1).
[13] Dwiardani K H, Sari L A, Wulansari P D, Nindarwi D D, and Arsad S 2020 IOP Conference Series: Earth and Environmental Science 441(1).
[14] Holy N H and Sari L A 2020 IOP Conference Series: Earth and Environmental Science 441(1).
[15] Sukenda, Dwinanti S, and Yuhana M 2009 Aquaculture 8(2) 2.
[16] Menteri Kelautan Dan Perikanan 2013 Keputusan Menteri Kelautan dan Perikanan Nomor 26 tentang Penetapan Jenis-Jenis Hama Dan Penyakit Ikan Karantina, Golongan, Media Pembawa, Dan Sebarannya (Jakarta: Kementerian Kelautan dan Perikanan) [in Indonesia].
[17] Afrianto E and Liviawaty E 1992 Pengendalian Hama dan Penyakit Ikan (Yogyakarta: Kanisius) [in Indonesia].
[18] Anggoro S 2005 J Fish Aquat Sci. 3(1) 61 p.
[19] Yuwono T 2006 Bioteknologi Pertanian Seri Pertanian (Yogyakarta: Gadjah Mada University Press) [in Indonesia].
[20] Sultana Y 2007 *Pharmaceutical Microbiology and Biotechnology Sterilization Methods and Principles* (New Delhi: Departement of Pharmaceutics Faculty of Pharmacy Jamia Hamdard) pp 2-5.

[21] Muharsini S, Wardhana, April H, and Suhardono 2003 *JITV* 8(4) 264-275 [in Indonesia].

[22] Surfianti O, Prihartini N C, Fathoni M, Ekoputri E R, Laminem, Wilis R, Pujiastuti E, Sokhib and Koeswara A D 2010 *Hemera Zoa Majalah Ilmu Keharianan Indonesia*.2(1) 15-24 [in Indonesia].

[23] Pranawaty R N, Buwono I D and Liviawaty E 2012 *JPK* 3(4) 61-74 [in Indonesia].

[24] Yuwono T 2006 *Teori dan Aplikasi Polymerase Chain Reaction* (Yogyakarta: Penerbit Andi) [in Indonesia].

[25] Badan Karantina Ikan Pengendalian Mutu Dan Keamanan Hasil Perikanan 2015 *Petunjuk Teknis Pemantauan Hama Dan Penyakit Ikan Karantina* (Jakarta: Badan Karantina Ikan Pengendalian Mutu Dan Keamanan Hasil Perikanan) [in Indonesia].

**6. Acknowledgment**

The authors are grateful to the Faculty of Fisheries and Marine, Airlangga University which had provided us the area and laboratory space to conduct this research. All authors approved the final draft of the manuscript, and we declare that there is no conflict of interest in all aspects of the work.