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PCR (Polymerase Chain Reaction) Method As A Detector of IMNV (*Infectious myonecrosis virus*) Disease in Vaname Shrimp (*Litopenaeus vannamei*)

Dela Shinta Maharani[1], Muhammad Sumsanto[1*]

[1]Aquaculture Study Program, Faculty of Agriculture, University of Mataram
Pendidikan Street No. 37 Mataram, West Nusa Tenggara

***Correspondence**:
muhammadsumsanto@unram.ac.id

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ABSTRACT

Indonesia is a maritime country that has great potential in the fisheries sector, especially in cultivating vaname shrimp (*Litopenaeus vannamei*). Disease in vaname shrimp is the biggest factor causing cultivation failure, one of which is caused by IMNV (*Infectious myonecrosis virus*). The aim of this research is to study IMNV detection techniques in vaname shrimp using the PCR (*Polymerase Chain Reaction*) method at the Situbondo Brackish Water Aquaculture Fisheries Center. This research uses a descriptive method, with stages starting from necropsy, RNA extraction using the silica method, amplification using RT-PCR and Nested-PCR, to electrophoresis and documenting the results using UV documentation. The results showed that the PCR technique was effective in detecting IMNV in shrimp, with DNA amplification clearly visible through agarose gel electrophoresis. Documentation of PCR results indicated the presence of a DNA band in the IMNV positive sample, which corresponded to the positive control. In conclusion, IMNV detection using the PCR method at BPBAP Situbondo has proven to be effective for identifying disease in vaname shrimp and can be applied as an initial step in controlling disease in vaname shrimp cultivation.

INTRODUCTION

Indonesia is a maritime country with 2/3 of its territory being ocean. Geographically, around 70% of Indonesia's territory is waters that have great potential to store extraordinary marine resources, ranging from fisheries potential, marine services, marine industry, transportation, to marine tourism (Suman *et al.*, 2016). Indonesian society has made many innovations and applied technology in exploring marine wealth, both capture fisheries and aquaculture. Aquaculture activities have a fairly high existence in society because they have a high and stable selling value, and very high export market demand is a special attraction. One of Indonesia's leading commodities is vaname shrimp.

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Vaname shrimp (*Litopenaeus vannamei*) originates from the subtropical areas of the west coast of America, from the Gulf of California in northern Mexico to the west coast of Guatemala, El Salvador, Nicaragua, Costa Rica in Central America to Peru in South America. Data from the Ministry of Maritime Affairs and Fisheries, shrimp production throughout 2022 reached 1,099,976 tons, an increase of 15% from 2021 and contributed 45.6% of Indonesia's export commodities (Munaeni *et al.,* 2023). The high market demand has made farmers increase the density of shrimp in ponds and the physiological nature that is susceptible makes shrimp very susceptible to disease. Disease attacks can cause mass mortality and significant losses for both extensive, semi-intensive and intensive cultivation. Initially, whiteleg shrimp were considered resistant to disease, but in fact these shrimp are very susceptible to disease.

Diseases in vaname shrimp are the biggest factor causing cultivation failure that must be overcome. In fact, these diseases are often the cause of mass death so that farmers experience significant economic losses and decreased production. External factors that cause disease such as viruses, bacteria, parasites, poor environmental conditions and pond management that is not in accordance with SOP (Standard Operating Procedure) Pathogens in the form of bacteria, viruses and parasites are infectious diseases that can cause death up to 60-80% if not handled properly. In addition, shrimp that have been infected with the disease will become carriers.

IMNV is an acute disease and causes quite high mortality, but this disease can also progress to a chronic disease with low mortality. The most common symptoms of the disease are, damage (necrosis) in the white cloudy color of the muscle/meat, especially in the upper abdominal muscles (back) and tail which appear opaque white to reddish (Novita *et al*., 2009). One way to identify IMNV is using PCR (Polymerase Chain Reaction). In the context of detecting viruses and bacteria, the PCR method has a high level of specificity and sensitivity compared to other diagnostic methods. One of the laboratory locations for identifying shrimp diseases and their treatment is the Brackish Water Aquaculture Center, Situbondo, East Java. his study was conducted with the aim of finding out how to identify diseases in shrimp using the PCR method.

METHODS

This research was conducted on August 2024 at the Brackish Water Aquaculture Center (BPBAP) Situbondo, East Java. This study uses a descriptive method (Suryana & Purwanto, 2019) with the aim of providing an overview of the procedure for identifying IMNV disease in shrimp using the PCR method carried out at the Brackish Water Aquaculture Center.

RESULTS

Necropsy

Necropsy samples are intended to take samples of target organs that are indicated by disease. For IMNV disease examination, the target organs and the number of samples needed are listed in the following table.

Table 1. Target Organs for IMNV Examination

Test Sample	*Target Organ*	*Amount/Volume*

Shrimp	striated muscle	5-10 tails are homogenized, take 20 mg
Nauplii and post larvae	Whole body	500 tails were homogenized, 20 mg was taken
Pond water	Centrifuge 12,000 rpm 5 minutes, 1 ml, 5 times	100 µl from 5 ml

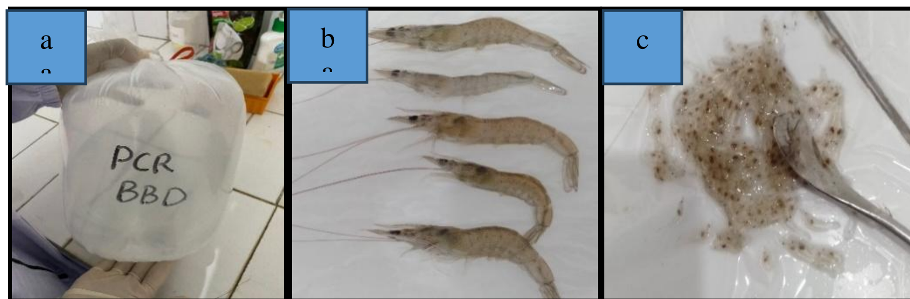

Figure 1. Sample of Vaname Shrimp
Description: (a) Sample Gasket, (b) Tocolan, (c) Post Larva

Extraction

Extraction is a process carried out to obtain pure DNA/RNA. The stages in the IMNV test sample extraction process are as follows;

1. Homogenize the sample using a spatula or scissors according to applicable provisions.
2. Weigh 20 mg sample into a 1.5 tube, add 900 µL GT buffer, homogenize the sample with a pellet pestle.
3. Centrifugation at 12,000 rpm for 3 minutes.
4. Prepare a new 1.5 mL tube, add 40 µL of silica
5. After centrifugation, transfer 600 µL of the supernatant into a new tube (provided in step 4).
6. Centrifuge at 12,000 rpm for 1 minute. Then discard the supernatant.
7. Add 500 µL GT buffer to wash the silica pellet. Vortex the pellet until completely dissolved (you can break the pellet with a pipette tip)
8. Centrifuge at 12,000 rpm for 1 minute. Discard the supernatant.
9. Add 1000 ML of 70% ethanol to wash the silica pellets. Vortex the silica pellets until completely dissolved (you can break the pellets with a pipette tip)
10. Centrifuge at 12,000 rpm for 1 minute. Discard the supernatant. Pipette off excess 70% ethanol.
11. Add 100 µL DEPC ddH20 to dissolve the SILICA pellet. Vortex the SILICA pellet until completely dissolved. Incubate at 55°C for 10 minutes. Vortex and centrifuge at 12,000 rpm for 2 minutes.
12. The DNA is ready for further application (or stored in a -20° C freezer until the DNA is used).

RNA amplification

Amplification is done with two PCR models, namely the first *Reverse Transcription* (RT-PCR) and the second is *Nested* -PCR. This is done because the IMNV virus is an RNA virus and

must be changed to cDNA so that it can be observed in the PCR process and the RT-PCR process functions to change RNA into cDNA. The amplification process begins with the creation of a mastermix for RT-PCR as follows:

Table 2. IMNV RT-PCR Reagent Volume

RNA Reagent	Volume (µl)
Sterile aquadest (NFW)	8,125
My Taq One-Step RT-PCR Kit	12.5
Primer 4587 Forward IMNV (5'-CGA-CGC-TGC-TAA-CCA-TAC-AA-3')	1
Primer 4914 reverse IMNV (5'-ACT-CGG-CTG-TTC-GAT-CAA-GT-3')	1
Reserve Tracriptase Enzyme	0.125
RNAse Inhibitors	0.25
Template	2
Total	25 µl/reaction

Table 3. Temperature Profile and Thermal Cycle of IMNV RT-PCR

Process	Temperature (^{0} C)	Time	Cycle
Reverse transcript	60	30 minutes	1
Denaturation	95	2 minutes	
Anneling	95	45 seconds	39
	60	45 seconds	
extension	72	7 minutes	1
Hold	4	∞	

Table 4. IMNV *Nested-PCR* Reagent Volume

RNA Reagent	Volume (µl)
Sterile aquadest (NFW)	8,125
My Taq HS Red Mix	12.5
Primer 4725 *Nested* Forward IMNV (5'-GGC-ACA-TGC-TCA-GAG-ACA-3')	1
Primer 4863 *Nested* reverse IMNV (5'AGC-GCT-GAG-TCC-AGT-CTT-G-3')	1
Template	2
Total	24.625 µl/reaction

Table 5. Temperature Profile and Thermal Cycle of *Nested* -PCR IMNV

Process	Temperature (^{0}C)	Time	Cycle
Denaturation	95	2 minutes	1
Anneling	95	30 seconds	39
	65	30 seconds	
	72	30 seconds	
Extension	72	2 minutes	1
Hold	4	∞	

Electrophoresis

Electrophoresis is a method of separating DNA fragments that utilizes an electric field to separate DNA fragments that move from the negative pole to the positive pole. The electrophoresis process uses 0.5x TAE Buffer Solution and inserts the amplicon into the agarose gel well that has been submerged in the electrophoresis machine and run for 25 minutes.

Figure 2. Preparation of 0.5x TAE solution

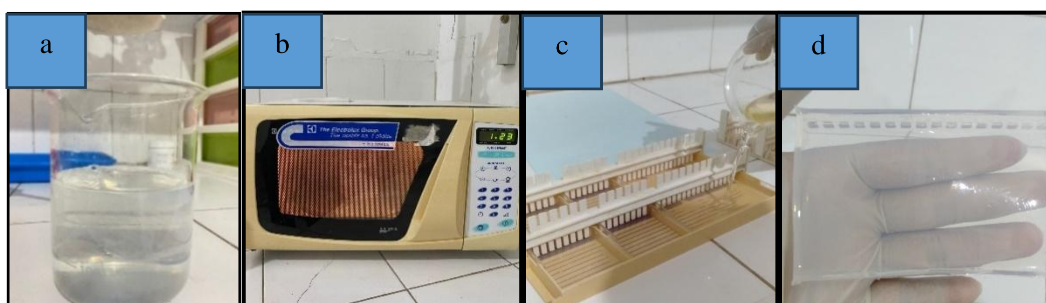

Figure 3. Making Agarose Gel
Information: (a) Agarose Gel Solution, (b) Agarose Gel Solution Heated in A Microwave, (c) Agarose Gel Printing, (d) Agarose Gel

Figure 4. Electrophoresis

Reading of Documentation Results

The results of reading PCR documentation using UV gel doc are as follows.

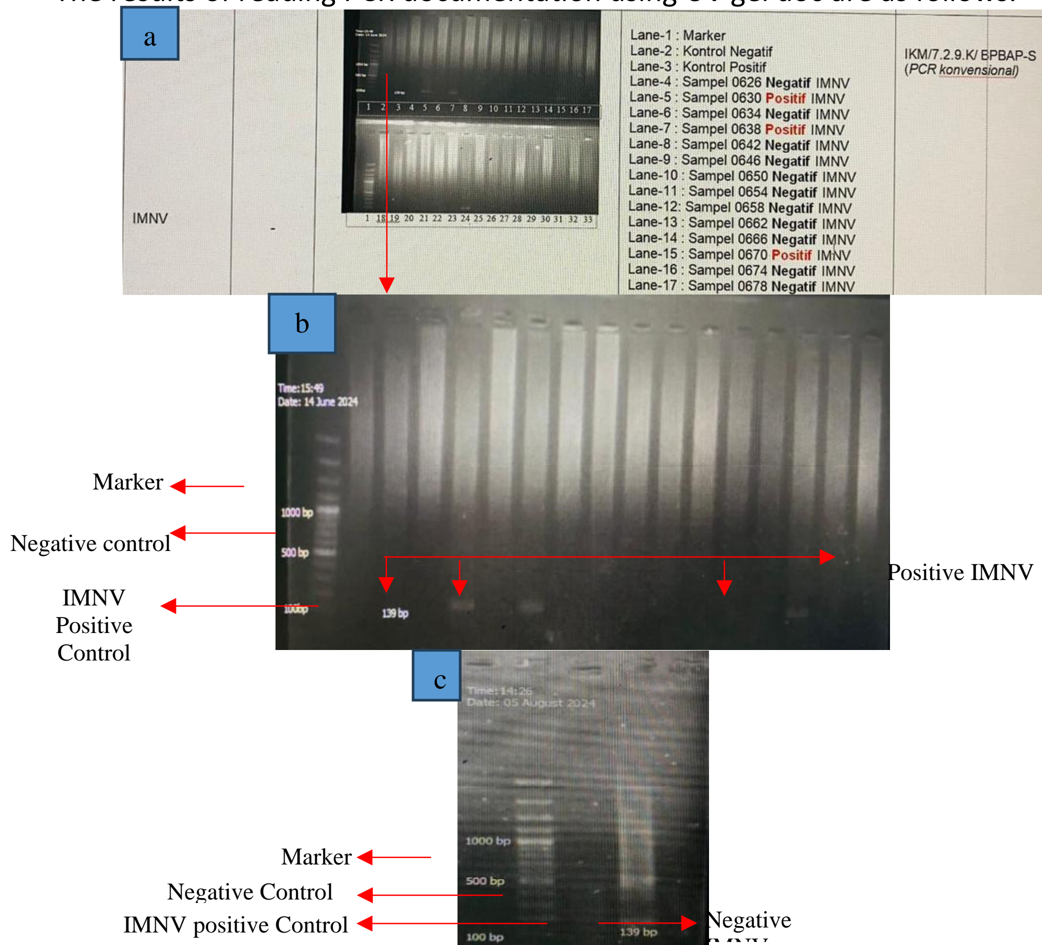

Figure 8. UV doc Documentation Results

DISCUSSION

Sample Necropsy

Sample necropsy was conducted in the Necropsy Room of the KESLING BPBAP Situbondo Lab. Sample preparation aims to take samples of target organs indicated by disease. Vaname shrimp samples consist of nauplii, post larvae (PL), tocolan/potential broodstock and broodstock. According to Sains *et al.* (2023), the nature of the necropsy results is based on changes in anatomical pathology. The IMNV virus affects the main muscles of the body. For the examination of IMNV disease, the target organs and the number of samples needed are listed in table 1.

The tools and materials needed are vaname shrimp, gloves, dissecting set, clear plastic, analytical scales, and 1.5 ml microtubes. Before sample preparation, sterilize all equipment such as scissors, tweezers, spatulas, with an autoclave and the table by spraying 70% alcohol. Each sample uses different equipment to avoid contamination.

The first stage is to prepare a 1.5 ml microtube to be labeled or coded in the form of numbers according to the sample sequence. The second stage is to prepare shrimp samples, for nauplii and PL, all parts of the body are ground, while for tocolan or shrimp older than 1 month, the striated muscle is taken and then ground until smooth. The third stage, the ground sample is then weighed as much as 20 mg with an analytical balance. The last stage, the sample

is put into 2 tubes, the first tube for extraction and the second tube as a sample retainer. After the preparation is complete, all equipment is washed with soap and dried, then autoclaved and the preparation table is cleaned with 70% alcohol.

Extraction

Extraction is a process carried out to obtain pure DNA/RNA. According to Ghabrial & Nibert (2009), IMNV disease in whiteleg shrimp is caused by a double-stranded RNA virus classified in the Totiviridae family with a diameter of 40 nm. Extraction aims to separate RNA from other cell components such as protein, fat, carbohydrates and body tissue. The entire extraction process is carried out in a laminar air flow so that it is necessary to sterilize the laminar room and extraction equipment with UV light for 15 minutes and turn on the fan mode to avoid contamination during the extraction process.

The first stage in the extraction is by adding 900 µl of the first GT buffer to lyse the cells so that the RNA can be extracted, then the sample is ground with a pastel pellet and then centrifuged at 12,000 rpm for 3 minutes. The centrifuge aims to separate the substrate based on the molecular weight. So that the sample sediment is obtained at the bottom of the microtube and the supernatant in the upper layer. The supernatant contains DNA, RNA, nucleic acids, proteins and others. The second stage, 600 µl of supernatant is added to a tube containing 40 µl of silica then vortexed and centrifuged at 12,000 rpm for 1 minute. According to Ariyanti & Sianturi (2019), Silica functions to bind DNA/RNA and separate DNA/RNA from impurities. The third stage, discard the supernatant, add 500 µl of the second GT buffer to lyse the remaining impurities attached to the silica, then vortex and centrifuge at 12,000 rpm for 1 minute. The fourth stage, discard the supernatant, add 1000 µl of 70% ethanol to wash the silica then vortex and centrifuge at 12,000 rpm for 1 minute. While waiting for centrifugation, the luminar air flow table is sterilized with 70% alcohol and prepare tissue to drain the remaining ethanol in the sample tube. The fifth stage, discard the supernatant and drain the tube by turning it upside down on the tissue. The sixth stage is resuspending the pellet with 100 µl of DEPC H_2O to dissolve the DNA that has been bound by silica, then vortex and incubate using a heating block at a temperature of 55 ° C for 10 minutes. After the incubation process is complete, the last stage is centrifugation at 12,000 rpm for 2 minutes and the genome is ready to be used for the amplification process.

PCR Amplification

The PCR method used in the BPBAP Situbondo Fish and Environmental Health Laboratory refers to the OIE (*Office International des Epizooties*) standard, the old name of the World Organization *for Animal Health (WOAH*). According to Setyawati & Zubaidah (2021), Amplification in the PCR method is the process of extending DNA segment strands in vitro using a thermal cycler machine. The amplification process will amplify DNA millions of times more so that it can be visualized in agarose gel electrophoresis.

Amplification is done with two PCR models, namely the first *Reverse Transcription* (RT-PCR) and the second is *Nested* -PCR. This is done because the IMNV virus is an RNA virus and must be changed to cDNA so that it can be observed in the PCR process and the RT-PCR process functions to change RNA into cDNA. The amplification process begins with the creation of a mastermix for RT-PCR in table 2 and table 3.

The RT-PCR process lasts for approximately 1 hour and 55 minutes, after the RT-PCR process (step 1) is complete the microtube is removed from *the thermal cycler* and continued with the *Nested* -PCR process (step 2). *Nested* -PCR is useful for multiplying target DNA fragments in large quantities according to the primers used, but the results of *Nested* -PCR DNA fragments are more specific (short) compared to regular PCR. Take 2 µ l template and

inserted into each tube containing *Nested mastermix* . Nested materials and cycles are listed in Table 4 and Table 5.

Reverse Transcription PCR (RT-PCR) The principle is to convert RNA into its complementary DNA sequence with *reverse transcriptase*, synthesize the second strand with DNA polymerase, and finally produce dscDNA molecules that can be amplified by PCR normally. This process occurs at a temperature of 60 ^{0}C for 30 minutes. The next stage is DNA template denaturation, namely the breakdown of double DNA strands into single strands. DNA template denaturation occurs at a temperature of 95 ° C for 1-2 minutes so that the double-stranded DNA *will* separate into single strands. Then continued with the process of attaching (annealing) the primer to the template DNA. The primer will form hydrogen bonds with the template in the complementary sequence area. The next stage is DNA extension where new DNA will be formed based on the information in the template DNA with the help of the DNA polymerase enzyme. This synthesis stage usually occurs at a temperature of 72 ° C for 1-2 minutes. After the process is complete, the thermalcycler is turned off and the microtube is removed and the PCR product (amplicon) is ready to proceed to the next stage, namely electrophoresis.

Electrophoresis

Electrophoresis is a method of separating DNA fragments that utilizes an electric field to separate DNA fragments that move from the negative pole to the positive pole. Electrophoresis requires a separating medium in the form of a stationary phase such as Agarose cells mixed with a buffer solution. According to Harahap (2018), TAE buffer solution functions to maintain the pH in the separating medium, and as a medium for providing electrolytes in the process of electric current movement. The process of diluting the 0.5x TAE Buffer solution ready to use, the first step is to take 50 ml of 10x TAE stock solution then dilute it in 950 ml of sterile aquadest and homogenize it by shaking it slowly. According to Sukartiningrum (2012), TAE Buffer Solution is made from a mixture of tris-base, glacial acetic acid, and EDTA pH 8. In molecular biology it is used for agarose electrophoresis because it can be a separation of DNA and RNA nucleic acids.

0.5x TAE Buffer solution is also used to make agarose gel. Agarose gel is a raw material in electrophoresis analysis to separate DNA, RNA, and protein molecules of various sizes. Agarose gel is a porous matrix that functions as a filter where nucleic acid molecules migrate. The first step is to weigh 3.69 grams of agarose powder and then put it into a beaker containing 300 ml of 0.5x TAE buffer, then homogenize it by stirring. The second step, the solution is heated in a microwave for the first 2 minutes and then homogenized and reheated 2x1 minute until boiling or clear. After that, the solution is removed from the microwave and cooled at room temperature until it reaches a temperature of 65 ° C. The third step, add 15 µl of florosafe. Florosafe is homogenized by rotating the beaker. According to Fadllan *et al.* (2019), Florosafe DNA stain is mixed when making agarose gel which functions to color DNA so that it can be seen when exposed to ultraviolet light. Florosafe is a DNA dye that has a specific wavelength that can capture the wavelength of DNA bands exposed to UV light by inserting into the DNA. After that, the agarose gel solution is poured into a thin Perspex sheet mold that is shaped like a comb and left for 30 minutes. The comb is inserted into the end of the gel that is still liquid. Thus, when the gel has solidified and the comb is lifted, small holes are formed which are used to insert DNA molecule samples.

The first step carried out during electrophoresis is that the printed gel is placed on an electrophoresis tank containing 500 ml of 0.5x TAE solution (until the gel is submerged). Then 5 µl of amplicon is inserted into the gel well and 3 µl of 100 bp marker. Markers are DNA

segments whose size specifications are known. Markers function as a reference to determine the size of the *amplicon* DNA band. After all amplicons are inserted into the gel well, close the electrophoresis machine and set the voltage to 100 volts for 25 minutes. Then lift the gel from the electrophoresis tank and document it with UV Documentation.

PCR Documentation

After electrophoresis, turn on the computer and turn on UV documentation (UV DOC). Lift the Gel from electrophoresis and insert it into UV Doc. Open the UV Doc application from the computer then select start exposure. Select stop exposure when the gel is visible on the monitor. The gel will be automatically photographed when stop exposure is selected. Observe the gel then provide the date and time. According to Khofifah *et al*. (2023), the UV Transilluminator in UV Doc functions to detect DNA bands from electrophoresis, so that DNA bands observed with UV light can be seen through the computer.

Reading of Documentation Results

Marker is a specific DNA segment and its size is known. Marker in PCR has a function as a reference to see the size of the amplified DNA in addition to functioning as a marker for the position of the base pair of the DNA molecule that migrates from the negative pole to the positive pole. The way to read the results is by observing the fluorescence line of the sample DNA band compared to the positive and negative control bands. The DNA band that is parallel to the positive control band indicates that the sample is positive for IMNV as shown in the image B *lane* -5, *lane-* 7 and *lane* -15. If there is no fluorescence of the DNA band on the line the same as the negative control, then the sample is declared negative for IMNV as shown in the image C *lane* -1. The DNA band can fluoresce due to the addition of fluorescence in the agarose gel and during amplification the IMNV specific primer binds the DNA band and multiplies the DNA segment up to millions so that it can be seen during electrophoresis.

CONCLUSION

Based on the Field Work Practices implemented, the IMNV identification technique carried out at BPBAP Situbondo is the PCR method. The first stage carried out in the PCR process is necropsy by taking the target organ infected with IMNV, namely the striated muscle part. The second stage is the extraction of sample RNA using the silica method which can bind IMNV virus RNA fragments. The third stage is the amplification process using the RT-PCR method to convert RNA into cDNA so that it can be amplified and continued with the *Nested* -PCR process to shorten the cDNA chain. The fourth stage is electrophoresis, and the fifth stage is documentation of the results using UV *documentation*.

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