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Rapid Detection and Identification of viral pathogens in fish aquaculture by PCR Protocols

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ABSTRACT

Fish diseases pose a universal threat to the ornamental fish industry, aquaculture, and public health. They can be caused by many organisms, including bacteria, fungi, viruses, and protozoa. Early detection of the causative agent which resulted in massive mortalities is a crucial aspect in the health management of fish aquaculture. Several diagnostic techniques have been developed for several pathogenic diseases and the use of molecular approaches is widely accepted. The present study optimized PCR protocols to detect a number of commercially important viral pathogens, including Infectious haematopoietic necrosis virus (IHNV), Viral haemorrhagic septicaemia virus (VHSV), Spring viraemia of carp virus (SVCV), Infectious salmon anaemia virus (ISAV), Koi herpes virus (KHV), Epizootic haematopoietic necrosis virus (EHNV), and other ranavirus. Red sea bream iridoviral and other megalocytivirus, viral nervous necrosis and other nodavirus (VNNV) in fishes as well as to ensure that these PCR assays are suited to Indian conditions. Many techniques have been developed, each requiring its own protocol, equipment, and expertise. In this regard, the advent of molecular biology, especially polymerase chain reaction (PCR), provides alternative means for detecting and identifying fish pathogens. The optimized PCR protocols for the detection of various viral pathogens in fish are sensitive as well as site-specific. These assays can be used as a management tool for the prevention and control of viral diseases in fish aquaculture in the country through early detection of the pathogen. In this review, recent advances in molecular fish pathogen diagnosis are discussed with an emphasis on nucleic acid-based detection and identification techniques. Major features and applications of current predominant methods and promising methods likely to impact future fish disease control and prevention are outlined.

Keywords: diseases, diagnostics, pond culture, virus, IHNV, VHSV, SVCV.

INTRODUCTION

The farming of fish and other aquatic animals is an ancient practice that is believed to date back at least 4000 years to pre-feudal China. Fish farms were common in Europe in Roman times. Bacterial and viral diseases are major problems in the fish aquaculture industry in the India. Disease has caused a significant drop in fish production resulting in tremendous losses among fish farmers. Among the major causes of fish diseases are luminous vibriosis due to *Vibrio* spp. and the viral nervous necrosis, which resulted in severe mortality of the cultured stock. Other viral pathogens that led to massive disease outbreaks include Infectious haematopoietic necrosis virus (IHNV), Viral haemorrhagic septicaemia virus (VHSV), Spring viraemia of carp virus (SVCV), Infectious salmon anaemia virus (ISAV), Koi herpesvirus (KHV), Epizootic haematopoietic necrosis virus (EHNV), other ranavirus, Red sea bream iridovirus , other megalocytivirus diseases and other nodavirus. Diseases due to these pathogens are usually encountered in hatchery and grow-out operations. A number of control measures used to curb these disease problems, including "green water" technology, properly drying the ponds during pond preparation, use of chlorinated reservoirs and treating the pond water prior to stocking, have to some extent help in controlling diseases in fishes. Important emerging viral pathogens of fish are found among many families of vertebrate viruses that are well-known to include pathogens of humans or domestic livestock. However, there are significant differences between the ecology of viral diseases of fish and those of humans or other terrestrial vertebrates. The most significant amongst these differences are that:

(i) Few fish viruses are known to be vectored by arthropods;

(ii) Water is a stabilizing medium, but currents are less effective for long range virus transmission than are aerosols;

(iii) Wild reservoir species are often at very low densities (except for schooling and aggregate spawning stocks);

(iv) Fish are poikilotherms and temperature has an exceptionally critical role in modulating the disease process by affecting both the replication rate of the virus as well as the host immune response and other physiological factors involved in resistance;

(v) Few fish viruses are transmitted sexually between adults, although high levels of some viruses are present in spawning fluids and a few viruses are transmitted vertically from adult to progeny, either intra-ovum or on the egg surface.

However, as occurs for avian diseases, migratory fish can serve as carriers for long-range dispersal of viral pathogens. The global expansion of finfish aquaculture and accompanying improvements in fish health surveillance has led to the discovery of several viruses that are new to science. Many of these are endemic among native populations and opportunistically spill-over to infect fish in aquaculture facilities. Other well-characterized fish viruses (e.g., channel catfish virus, *Onchorhynchus masou* virus) can also cause significant losses in aquaculture but do not seem to be increasing significantly in host or geographic range. Because of the

risk of spread through commercial trade in finfish, many of the diseases are listed as notifiable by the World Organization for Animal Health (OIE).

The use of molecular techniques over traditional methods of diagnosing fish diseases has been widely accepted because of its high degree of specificity and sensitivity, rapidity and its ability to detect the presence of pathogens even in extremely low amounts. Rapid detection can reduce disease risks and in the long run lead to enhanced production.

Degenerate polymerase chain reaction (PCR) primers and specific PCR conditions have been developed for the selective amplification of polymorphic regions. This specific multilocus PCR approach provides a powerful counterpart to random amplification of polymorphic DNA (RAPD) and restriction fragment length polymorphism (RFLP) analysis for the identification of causitive agents of diseases**.** Conventional PCR assays have been developed to detect pathogens in fishes. Despite its high cost due to expensive reagents and equipment, the amount is still insignificant in comparison to the amount saved when immediate management procedures can be applied to curb the spread of the disease and avoid mass mortality. In addition, PCR assays need to be optimized to suit the prevailing conditions where the biological samples were obtained. This is to prevent variations in the results of the assays, which can lead to misinterpretation, thereby causing severe consequences in the management of the aquaculture facility. PCR is one of the technologies that not only made a tremendous impact on the scientific community, but also affected many aspects of our everyday lives. PCR is a rapid, inexpensive and simple way of copying specific DNA fragments from minute quantities of source DNA material, even when that source DNA is of relatively poor quality. It does not necessarily require the use of radioisotopes or toxic chemicals. , it is a chain reaction, a small fragment of the DNA section of interest needs to be identified which serves as the template for producing the primers that initiate the reaction. One DNA molecule is used to produce two copies, then four, then eight and so forth. This continuous doubling is accomplished by specific proteins known as polymerases, enzymes that are able to string together individual DNA building blocks to form long molecular strands. To do their job polymerases require a supply of DNA building blocks, i.e., the nucleotides consisting of the four bases adenine (A), thymine (T), cytosine (C) and guanine (G). They also need a small fragment of DNA, known as the primer, to which they attach the building blocks as well as a longer DNA molecule to serve as a template for constructing the new strand. If these three ingredients are supplied, the enzymes will construct exact copies of the templates.

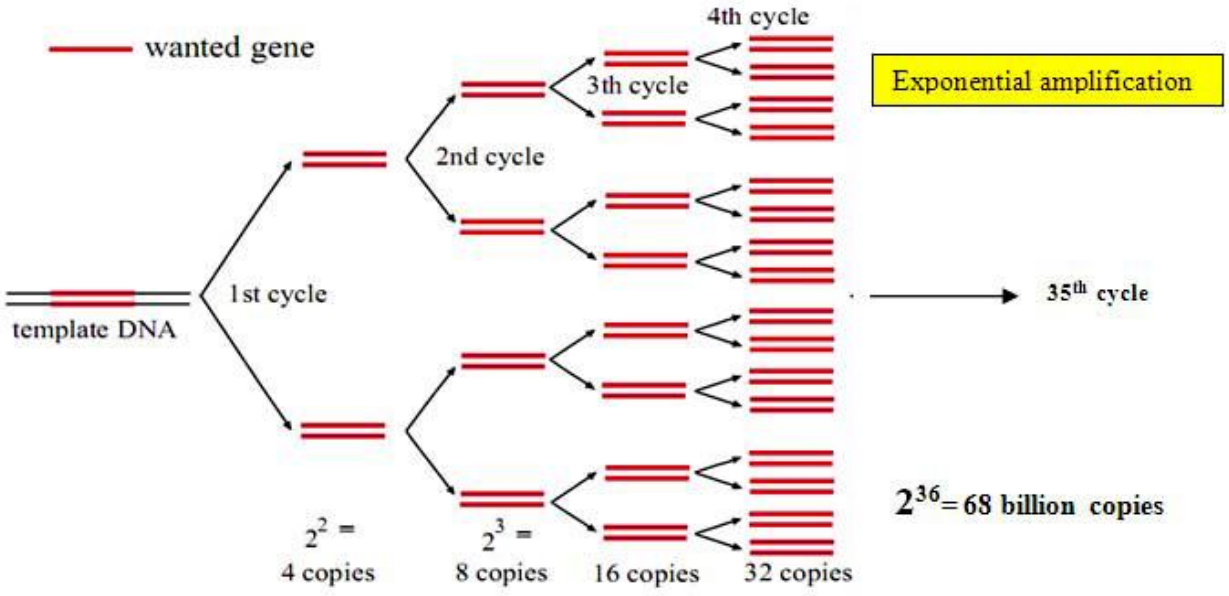

Fig1:

Number of copies of DNA obtained after 'n' cycles = 2(n+1)

It is then possible to clone DNA whose sequence is unknown. This is one of the method's major advantages. Genes are commonly flanked by similar stretches of nucleic acid. Once identified, these patterns can be used to clone unknown genes - a method that has supplanted the technique of molecular cloning in which DNA fragments are tediously copied in bacteria or other host organisms. With the PCR method this goal can be achieved faster, more easily and above all in vitro, i.e., in the test-tube. Moreover, known sections of long DNA molecules, e.g. of chromosomes, can be used in PCR to scout further into unknown areas.

Hence, this study was conducted for rapid detection and identification of viral pathogens in fish aquaculture by PCR Protocols in order that these will be suited to the existing conditions of the aquaculture sites in the India.

MATERIALS AND METHODS

Sampling of fish

Different stages of Salmonella fish were collected from fish farms and hatcheries in various fish producing regions in the Haryana. These places included: Punjab, Uttar Pradesh, Baroda, Mysore, Hyderabad and bheries of West Bengal and pokkali fields of Kerala, Madras, the Central Institute on Freshwater Aquaculture (CIFA) at Bhubaneswar, the Central Institute of Brackishwater Aquaculture (CIBA) at Chennai and the National Research Centre on Coldwater Fisheries (NRCCWF) at Bhimtal in Nainital, Lohit district of Arunachal Pradesh. The fishes were immediately dissected and tissue samples were placed in 1 ml DNA extraction buffer at room temperature. Remaining undissected fish samples were placed in ice and upon arrival in the laboratory stored at -80°C.

Extraction of DNA

Different tissues of shrimps were used for DNA extraction. Salmonella fish tissues were dissected and placed in 1 ml of DNA extraction buffer with the following composition; 10 mM Tris, 125 mM NaCl, 10 mM EDTA, 0.5% SDS and 4M Urea.

For the postlarvae samples, an additional step of eye stalk removal was employed to remove inhibitors. Proteinase K at a volume of 10 µl (1 mg/ml) was added to the extraction buffer and the solution was incubated for 1 hour at 37°C. Total DNA was extracted using phenol: chloroform: isoamyl alcohol. The aqueous (upper) layer (approximately 500 µl) was transferred to a new tube and mixed with an equal volume of absolute ethanol, 10 µl of 3M sodium acetate and 2 µl of 1% glycogen (1 mg/ml). The mixture containing the DNA was stored in the freezer (-20°C) for 24 hrs and then centrifuged at 12,500 rpm for 10 minutes at 4°C. The supernatant was discarded leaving the visible (precipitate) pellet in the tube. The DNA pellet was washed with 1 ml 70% ethanol and centrifuged at 12,500 rpm for 5 minutes at 4°C. The ethanol was discarded and the pellet was air dried for few minutes. Dried pellet was resuspended in 100 µl of 1X TE buffer and stored at -20°C until use.

Optimization of the PCR assays

The PCR primers for the detection of IHNV, VHSV and SVCV that were used in the present study were obtained from previously published primer sequences. New primers specific for isolate of IHNV were designed from the least conserved region of the partial sequence of the IHNV genomic DNA and amplified a fragment size of 193-bp.

PCR was performed following published protocol with some modifications. Optimization of the PCR assays was done by testing various annealing temperatures. A 20 µl reaction mixture was prepared containing 2µl of 10X PCR reaction buffer, 1.5µl of 50mM $MgCl_2$, 0.5µl of 10mM dNTP mix, 0.1µl of Taq Polymerase, 2µl each of the forward and reverse primers, distilled water, and DNA sample . The PCR amplification was performed with an initial denaturation at 95°C for 3 minutes and followed by 40 cycles of denaturation at 95°C for 30 seconds, annealing for 30 seconds at various temperatures for each primer set and elongation at 72°C for 1 minute. The PCR amplification was completed using a final elongation step at 72°C for 5 minutes then incubation at 4°C.

Five microliters (5 µl) of PCR product in 5µl 1X TE Buffer and 1 µl GLB Dye and 0.5µl of 50 bp DNA Ladder in 9.5µl 1X TE Buffer plus Dye were loaded into a 1.2% agarose gel with ethidium bromide and electrophoresed at 100V for 30-32 minutes. The bands were viewed using Gel Documentation System.

Ten-fold serial dilutions of the genomic DNA were prepared to determine the sensitivity of the PCR assays.

RESULTS AND DISCUSSION

The PCR assays for the detection of the various viral pathogens in fish aquaculture were optimized so that these will be applicable to Indian conditions. Published PCR primers were used for these assays. For the detection of IHNV, the primers developed by Surachetpong et al. were initially used for detection of the virus (Table 1). In a later study, partial sequence of strain of IHNV had 80% sequence similarity with other IHNV strains, indicating the need to develop strain-specific IHNV primers for detection of this pathogen.

New set of IHNV primers were designed for the specific detection of IHNV strain (see Table 1; for the primer sequence). The new primers generated a PCR product of 193 bp. Sensitivity of the new IHNV primers was compared to the existing primers used by Surachetpong et al. and Belcher and Young. The new primers were equally as sensitive as the previously published primers in the detection of IHNV in fishes. In addition, the new primers for the detection of the local strain of IHNV were highly specific for the virus because no cross amplifications were observed for other fishes viruses including, VHSV, SVCV, KHV, VNNV and EHNV. Several primers were used to detect the presence of VHSV in fish samples. For example, the primers developed by Flegel were able to detect the presence of the VHSV in diseased and asymptomatic adult fish and post larvae obtained from various localities (Fig. 2).

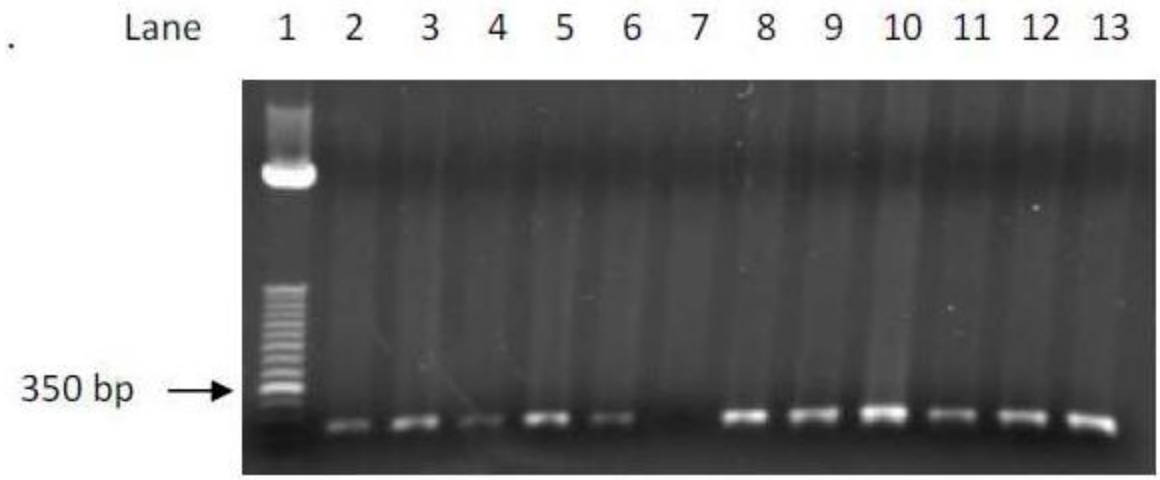

Fig. 2: Detection of VHSV in shrimp from various sites using published primers by Flegel at different annealing temperatures

Lane 1: 100 bp DNA marker; lanes 2 and 8: 55°C; lanes 3 and 9: 56°C; lanes 4 and 10: 58°C; lanes 5 and 11: 60°C; lanes 6 and 12: 61°C; lanes 7 and 13: 63°C

Published protocols for VHSV detection had to be modified when applied to detection of the pathogen in fish samples. A comparison of published protocols and the protocols that were developed in the study for VHSV detection is shown in Table 1. Most of the modifications were done on the annealing temperature and the time for each step during the amplification process. Lower annealing temperatures were used to obtain PCR products. These modifications in PCR conditions imply some differences in Indian strain of VHSV relative to the strains that are found in other countries. Local samples of post larvae were subjected to PCR to detect the

presence of SVCV using published primers by Yang et al. The said primers amplified a 703bp fragment of the capsid protein gene of SVCV. Amplification at different annealing temperatures showed the most distinct band, obtained at 65°C (Fig. 3).

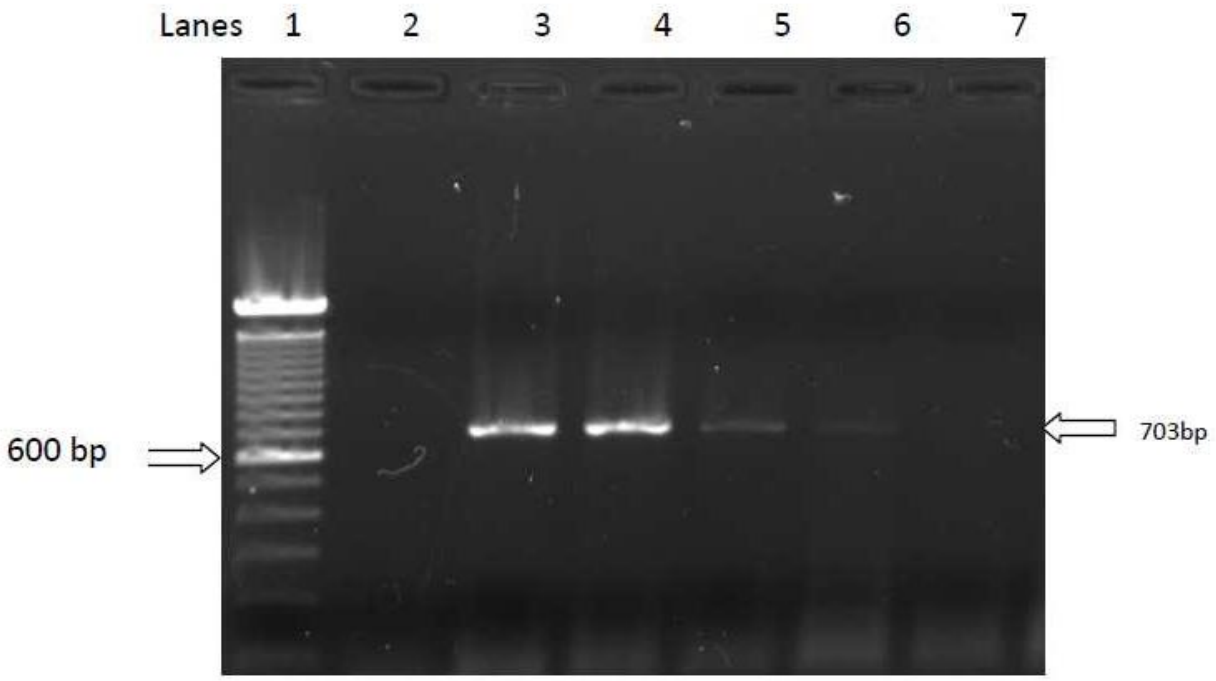

Fig. 3. Detection of SVCV from shrimp samples and determination of the optimum annealing temperature
Lane 1: 100 bp DNA ladder; lane 2: negative control; lane 3: 57°C; lane 4: 60°C; lane 5: 63°C; lane 6: 65°C

The PCR conditions that were used are shown in Table 1. The SVCV primers were able to detect the presence of SVCV in 10 pg DNA samples, although the electrophoresis band was very light. This DNA concentration was much higher. The difference in the sensitivity of the assays can be attributed to the fact that we used total DNA from fish post larvae that were infected with the virus. It was noted however that annealing temperature used in the study at 65°C was more stringent than the annealing temperature. Tang and Lightner have shown low sequence variability of SVCV isolates, and the strain that is prevalent in the Americas is closely related to the strain that was detected in the India, hence the published primers were suited to detect the local strain of the virus. Attempts to detect the presence of KHV in fish samples using the published primers did not produce any PCR product. This may imply that the fish in the wild and in the farms or hatcheries could be free from this virus or the viral load is too low to allow detection of the pathogen.

Table1. PCR primers and optimized amplification conditions for the detection of various viral pathogens of fishes in the India.

Pathogen/Primer Sequences	**Published PCR Conditions**	**PCR Conditions for Samples**
Infectious haematopoietic necrosis (IHNV), IHNV F- 5' GTACGGCAATACTGGAGGAGGT-3' IHNV R 5'-GGAGATGTGTAAGATGGACAAG-3' Product : 232 bp	No initial denaturation 40 cycles 95°C, 30s 60°C, 30s 72°C, 30s Final elongation: 72°C, 7min Incubation 4°C	Initial denaturation 95°C, 3min 40 cycles 95°C, 30s 58°C, 30s 72°C, 1min Final elongation: 72°C, 5min Incubation 4°C
IHNV1 5'-TGA TTC TGC ATC CAG CTC-3' IHNV2 5'- GCA CGG TCA ACA TGT CT-3' Product : 824 bp	Initial denaturation 94°C, 5min 30 cycles 94°C, 30s 56°C, 30s 72°C, 1min Final elongation: 72°C,5min Incubation 4°C	Initial denaturation 95°C, 5 min 30 cycles 95°C, 30s 52°C, 30s 72°C, 1min Final elongation: 72°C, 5min Incubation 4°C
Viral haemorrhagic septicaemia(VHSV), VHSV F 5'-AATCCTAGGCGATCTTACCA-3' VHSV R 5'-CGTTCGTTGATGAACATCTC-3' Product : 261 bp	94°C, 5min (hot start) 35 cycles 94°C, 30s 60°C, 30s 72°C, 30s Final elongation: 72°C,7min Incubation 4°C	Initial denaturation 95°C, 3min 40 cycles 95°C, 30s 550C, 30s 72°C 1min Final elongation: 72°C, 5min Incubation 4°C
VHSV F 5'-TCCAATCGCGTCTGCGATACT-3' VHSV R 5'-CGCTAATGGGGCACAAGTCTC-3' Product size: 361bp	Initial denaturation 96°C,5min 40 cycles 94°C, 30s	Initial denaturation 95°C, 3min 40 cycles 95°C, 30s

	65°C, 30s 72°C 1min Final elongation: 72°C,7min Incubation 4°C	570C, 30s 72°C 1min Final elongation: 72°C, 5min Incubation 4°C
Spring viraemia of carp(SVCV) SVCV F 5'-TAATGAAGACGAAGAACACGCCGAAGG-3' SVCV R 5'-TGGGTAGACTAGGTTTCCAAGGGATGGT-3' Product : 703 bp	94°C, 5min (hot start) 40 cycles 94°C, 1min 57°C, 1min 72°C, 1min Final elongation: 72°C, 5min Incubation 4°C	Initial denaturation 95°C, 5min 40 cycles 94°C, 1min 65°C, 1min 72°C, 1min Final elongation: 72°C, 5min Incubation 4°C
Monodon Baculovirus (MBV) MBV F 5'-TCCAATCGCGTCTGCGATACT-3' MBV R 5'-CGCTAATGGGGCACAAGTCTC-3' Product size: 361bp	96°C, 5min (hot start) 40 cycles 94°C,30 s 65°C, 30s 72°C, 1min Final elongation: 72°C,5min Incubation 4°C	Initial denaturation 95°C, 3min 40 cycles 94°C, 30s 57°C, 30s 72°C, 1min Final elongation: 72°C, 5min Incubation 4°C

CONCLUSION

In conclusion, this study has optimized protocols for the PCR detection of the commercially important viral pathogens of shrimp in the India. Each PCR assay has been tested and is deemed to be sensitive as well as site-specific. These assays can be used for routine procedures in shrimp health or diagnostic laboratories as a management tool for the prevention and control of viral diseases in shrimp aquaculture in the country through early detection of the pathogen. This study is part of the research project, "Biotechnology for Shrimp: Utilizing Molecular Technologies to Elucidate Shrimp Immunity and Develop Disease Diagnostics."

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