NOTE

Detection of marine birnavirus genome in zooplankton collected from the Uwa Sea, Japan

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ABSTRACT: Marine birnaviruses (MABVs) infect a wide range of fish and shellfish, yet their mode of transmission is still unclear. To determine whether marine plankton serve as a vector for MABVs, we examined plankton collected from the Uwa Sea, Japan. The phytoplankton and zooplankton were collected monthly, at depths of 0 and 40 m, from May to November 2001. Detection of the MABV genome was carried out using 2-step PCR and virus isolation. Viral genome was detected in zooplankton collected at 0 m depth in September and at 40 m depth in November. The virus could not be isolated in the PCR-positive samples. These results suggest that zooplankton may act as a vector of MABVs, although the infective and/or accumulated virus titer in zooplankton was low.

KEY WORDS: Birnavirus · Plankton · Reservoir · Vector · Uwa Sea

The marine birnavirus (MABV) group, a member of the *Birnaviridae*, are icosahedral unenveloped viruses whose genomes comprise 2 segments of double-stranded RNA designated A and B (Dobos et al. 1979). One example of MABV is the Yellowtail ascites virus (YAV), first isolated from diseased yellowtail *Seriola quiqueradiata* in Japan in 1985 (Sorimachi & Hara 1985). Since then, other similar birnaviruses have been isolated from many diseased fish and shellfish around the world (Castric et al. 1987, Sohn et al. 1995, Suzuki et al. 1998). Jung et al. (2001) reported that an MABV was isolated from diseased sea squirts *Halocynthia roretzi* in Korea. These findings suggest that MABVs have a broad host range among marine organisms. We suspect that plankton may serve as a vector of MABVs, because plankton are the food of most juvenile fish and shellfish. However, the mode by which MABVs are transmitted remains unclear. It is important to identify this mode in order to prevent MABV-caused diseases. This study aims to determine if MABVs are present in plankton.

Materials and methods. *Monitoring of basic physico-chemical and biological data:* We sampled plankton at a single sampling station in Uchiumi Bay, on the west coast of Ehime Prefecture, Shikoku Island, Japan, from May to November 2001. Water temperature and salinity were measured using a Chlorotech (Arec Electronics ACL- 208-DK) profiler. Chlorophyll *a* was measured according to Moran & Porath (1980). Plankton were collected between 0 and 2 m depth, and between 40 and 45 m depth, using 2 plankton nets of 20 μm and 200 μm, respectively. Samplings were performed 40 times at each depth. Zooplankton were sorted out under a stereomicroscope. This procedure was repeated twice. The dominant phytoplankton and zooplankton were monitored monthly.

Two-step PCR: RNA extraction and PCR were performed according to Suzuki et al. (1997a). Briefly, collected plankton were washed with artificial seawater 3 times and dried, then 400 μg plankton was homogenized and suspended in 45 μl TE (0.2 M Tris HCl, pH 8.3, 0.1 M EDTA) buffer. Five μl proteinase K (10 mg ml^{-1}, TaKaRa) was added. The mixture was incubated at 55°C for 2 h. Nucleic acids were extracted using the phenol-chloroform method. The extracted nucleic acid was heated at 100°C for 5 min with the addition of a primer set of P1-P2 (P1; 5′-AGAGATCACTGACTTCA-CAAGTGAC-3′, and P2; 5′-TGTGCACCACAGGAAA GATGACTC-3') following reverse transcription (RT) performed with Moloney murine leukaemia virus reverse transcriptase (MMLV, GIBCO) at 37°C for 1 h. To inactivate the reverse transcriptase, the sample was heated at 100°C for 5 min. The synthesized cDNA was employed for PCR. PCR amplification was performed in a DNA thermal cycler (TaKaRa) with 30 amplification cycles (95°C for 1 min, 50°C for 1 min, and 72°C for 1 min). A 5 μl portion of the RT-PCR product was employed for nested-PCR. The nested amplification with 30 cycles was conducted at 95°C for 1 min, 48°C

Table 1. Basic physico-chemical and biological data at 0 and 40 m depth at the sampling station in 2001

| | Water temp. (°C) | | Salinity (mg l^{-1}) | | Chl a (µg l^{-1}) | | Dominant phytoplankton | | Dominant zooplankton | |
	0 m	40 m	0 m	40 m	0 m	40 m	0 m	40 m	0 m	40 m
May	22.64	20.39	34.20	34.30	3.47	0.38	*Chaetoceros*	*Chaetoceros*	*Noctiluca*	*Oithona*
July	26.97	20.99	33.60	34.21	0.40	0.26	*Chaetoceros*	*Chaetoceros*	*Paracalanus*	*Paracalanus*
September	26.17	26.30	32.73	33.75	0.79	0.49	*Chaetoceros*	*Chaetoceros*	*Oithona*	*Oithona*
November	19.94	19.91	34.22	34.21	0.54	0.45	*Thalassiosira*	*Thalassiosira*	*Oithona*	*Oithona*

for 1 min, and 72°C for 1 min with a primer set of P3-P4 (P3; 5'-CAACACTCTTCCCCATG-3', P4; 5'-AGAAC-CTCCCAGTGTCT-3'). The purity and size of the amplified products were assessed using electrophoresis on 2% agarose gel. The gel was stained with ethidium bromide and visually examined under UV light.

Nucleotide sequencing: To perform nucleotide sequencing, the amplified PCR fragment (168 bp) was cut from the gel under the long wavelength of UV and was purified using Ultrafree-DA (Millipore). Sequencing was performed based on the dideoxy nucleotide termination method (Sanger et al. 1977) in an automated ABI PRISM 310 DNA sequencer (PE Biosystems) using the ABI PRISM BigDye Terminator Cycle Sequencing FS Ready Reaction Kit (PE Biosystems) and primer set of P3-P4 described above. Analysis of the results was performed using Genetyx Mac Version 8.0 software. The sequencing was carried out at the Center for Gene Research, Ehime University, Japan.

Cell culture: The chinook salmon embryo cell line (CHSE-214) was used for isolating the MABV in the PCR-positive samples. The cells were grown at 20°C in Eagle's minimum essential medium (MEM, Nissui)

containing 10% fetal bovine serum (FBS). Plankton from PCR-positive samples were homogenized in a 1000-fold volume of MEM, and the homogenate was centrifuged at $3000 \times g$ at 4°C for 15 min. The supernatant was filtered through a 0.45 µm pore filter (Millipore). A 500 µl portion of the filtrate was inoculated to a monolayer of CHSE-214 cells cultured in a 25 cm^2 flask (Corning), which were incubated at 20°C for 2 wk. The cells were monitored daily for cytopathic effect (CPE). When CPE was not observed, blind passage was performed.

Results and discussion. Basic physico-chemical and biological data collected at the sampling station are shown in Table 1. Water temperature reached a maximum in July at 0 m depth, and thermal stratification developed between May and July. The water temperatures in September and November were isothermal, probably due to vertical mixing. Salinity was almost constant during the study period. Chlorophyll a concentrations were highest at 0 m depth in May. The dominant phytoplankton during the experimental period was *Chaetoceros* spp., except in November, when *Thalassiosira* spp. was dominant. In September and November, the dominant zooplankton was *Oithona* spp.

The results of testing for the MABV genome by 2-step PCR in the plankton are shown in Fig. 1. Positives were not detected from phytoplankton. MABV genome was detected in zooplankton collected at 0 m depth in September and 40 m depth in November. In this experiment, the PCR-positive sample was only 2 out of 8 zooplankton, which is a low positive rate. However, we obtained similar data in 1999. Zooplankton were collected at the same station in July, September, October and November 1999. MABV genome was detected in zooplankton collected in September and November. The PCR-positive sample was 4 out of 12 (33.3%) zooplankton. This additional supporting evidence leads us to conclude that MABV either infects or is concentrated in zooplankton. The seawater containing PCR-positive plankton
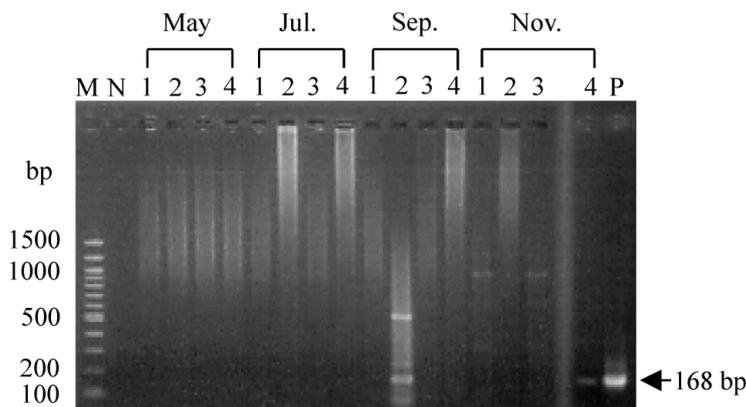

Fig. 1. Detection of the marine birnavirus (MABV) genome by 2-step PCR in plankton collected at 0 to 2 m and 40 to 45 m depth. Lanes: M, molecular marker; N: negative control without template; (1) phytoplankton collected at 0 to 2 m; (2) zooplankton collected at 0 to 2 m; (3) phytoplankton collected at 40 to 45 m; (4) zooplankton collected at 40 to 45 m; P: positive control; MABV Y-6 strain, isolated from yellowtail

(A)

VP2	NS	VP3

◄────► RT-PCR

◄────► Nested-PCR

(B)

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PCR product  GCGGCACCAC TCATCGGAGC CGCCGACCAA TTCATCGGAG ACCTGACCAA      50
JPO-96       ---------- ---------- ---------- ---------- ----------      50
AGJ-90       ---------- ---------- ---------- --------G- ----------      50
Y-6          ---------- ---------- ---------- ---------- ----------      50
AY-98        ---------- ---------- ---------- ---------- ----------      50


PCR product  GACCAACGCA GCCGGAGGCC GCTACCTAAC ACATGCAGCA GGAGGACGCT     100
JPO-96       ---------- ---------- ---------- ---------- ----------     100
AGJ-90       ---------- ---------- ---------- ---------- ----------     100
Y-6          ---------- ---------- ---------- ---------- ----------     100
AY-98        ---------- ---------- ---------- ---------- ----------     100


PCR product  ACACTGACGT GATGGACTCC TGGGCTAGCG GCAC                      134
JPO-96       ---------- A--------- ---------- ----                      134
AGJ-90       ---------- A--------- -----C---- ----                      134
Y-6          ---------- A--------- -----C---- ----                      134
AY-98        ---------- A--------- -----C---- ----                      134
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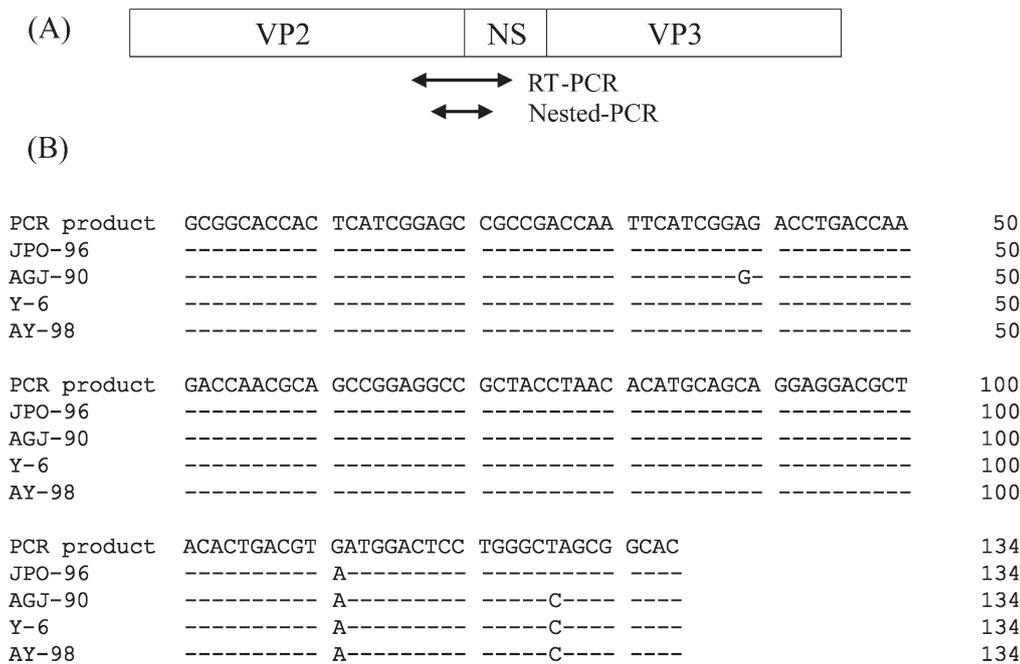
Fig. 2. (A) Map of segment A of aquabirnavirus and the PCR region. (B) Nucleotide sequences of the 2-step PCR products and the isolated strains. Sequences obtained in September and November were the same. Strains JPO-96, AGJ-90, Y-6 and AY98 were used for comparison. – : same base as in PCR-product

was filtered to remove plankton. PCR was then employed to confirm that the seawater itself was not origin of the virus. MABV genome was not detected in the seawater, which indicates that the MABV genome originated in plankton. Furthermore, the large band was observed in the September sample (Fig. 1, lane 'Sep. 2'). This band was sequenced, but no homologous sequences were found in DDBJ. This indicates that the band is non-specific, probably derived from plankton DNA.

This is the first report about MABV genome detected in identified zooplankton. White spot syndrome virus (WSSV), which is an important pathogen in shrimp, can infect copepods (Lo et al. 1996), and fish nodavirus can infect zooplankton such as artemia *Salina nauplii* and the rotifer *Brachionus plicatilis* (Skliris & Ricards 1998). Since both viruses are crustacean viruses, it is reasonable that they infect crustacean plankton. MABVs have a broad host range, which includes fish, molluscan shellfish and protochordates, but are not known to infect crustaceans. Still, it is not clear whether the MABV infected or was accumulated in zooplankton. The virus infectivity titer in plankton should be low, because the virus was not isolated from PCR-positive zooplankton (data not shown). MABV genome was detected in September and November, but not in May and July. This suggests that MABV

infection and/or accumulation in zooplankton varies seasonally. It is thought that zooplankton containing the MABV group increased in abundance from autumn to winter in the Uwa Sea. The habitat depth of the infected zooplankton also varied; the viral genome was detected in zooplankton collected at 0 m in September, and at 40 m depth in November. Zooplankton such as copepods show patchy distribution in the water column and diel vertical migration. It is therefore possible that we sampled the zooplankton from a patch.

The junction region between the genes VP2 and NS (Fig. 2A) is variable, but useful for the genogrouping of aquabirnaviruses (Heppell et al. 1992, 1993, Hosono et al. 1996). The sequence data from the present study were compared with some MABV strains isolated from fish and shellfish around western Japan (Fig. 2B). The sequences of the 2nd PCR-positive samples were the same. When compared with all reference strains, the 111th base was G, although there was no amino acid change. The difference between JPO-96 (Suzuki et al. 1998) isolated from the Japanese pearl oyster and the PCR-product in this study was only at the aforementioned 111th base. Also, AGJ-90 (Suzuki et al. 1997b) isolated from the agemaki (jack knife clam) *Sinonovacura constrica* showed that the 39th base was G and the 126th base was C, whereas these were A and T in the present study. Fish origin strains Y-6 (Hosono et al.

1996) isolated from the yellowtail and AY-98 (Jung et al. 1999) isolated from the ayu *Plecoglossus altivelis* had C at the 126th base, whereas the PCR products in our study had a T at this base. The highly conserved sequences among the MABV strains suggest that MABVs occurring in western Japan might be genetically similar.

In summary, MABV genome was detected in zooplankton. The number of zooplankton containing the MABV group increased from autumn to winter. The sequence analysis of the MABV genome in the plankton was similar to those of MABVs isolated from western Japan in previous studies. These results suggest that zooplankton may serve as a vector of MABVs in these areas. The infective state of MABVs in zooplankton will be further examined in the future.

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