天然ニホンウナギ親魚からのJapanese eel endothelial cells-infecting virus(JEECV)の検出

誌名	魚病研究
ISSN	0388788X
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発行元	日本魚病学会
巻/号	51巻2号
掲載ページ	p. 64-66
発行年月	2016年6月

農林水産省 農林水産技術会議事務局筑波産学連携支援センター
Tsukuba Business-Academia Cooperation Support Center, Agriculture, Forestry and Fisheries Research Council Secretariat
AgriKnowledge

魚病研究 **Fish Pathology, 51 (2), 64–66, 2016. 6**

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Short communication

Detection of Japanese Eel Endothelial Cells-infecting Virus (JEECV) in Mature Japanese Eel *Anguilla japonica* Caught from Their Spawning Area

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(Received January 26, 2016)

ABSTRACT—We examined the infection of Japanese eel endothelial cells-infecting virus (JEECV), which is the agent of viral endothelial cells necrosis (VECNE), in Japanese eels caught from the southern part of West Mariana Ridge in 2013 to know the infection status of the virus in their spawning area. JEECV was detected in the gills from a female out of five mature male and female fish by both quantitative and conventional PCRs. Additionally, the predicted polyomavirus large T like protein of the detected virus was different by one amino acid from that of the virus from farmed eels with VECNE.

Key words: Japanese eel endothelial cells-infecting virus, JEECV, VECNE, spawner, *Anguilla japonica*

The Japanese eel *Anguilla japonica* is an important fish species in East Asia. Since the 1980s, viral endothelial cell necrosis of eel (VECNE) has caused serious damage to eel farms (Egusa *et al*., 1989; Kusuda *et al*., 1989; Ono *et al*., 2003; Ono *et al*., 2007; Mizutani *et al*., 2011), thereby posing a threat to the Japanese eel population. VECNE is caused by infection with the Japanese eel endothelial-cells infecting virus (JEECV) (Mizutani *et al*., 2011). JEECV can be classified as a member of the family Polyomaviridae, because an open reading frame (ORF) of JEECV is homologous to the large T antigen gene of polyomaviruses (Mizutani, *et al*., 2011). Since the T antigen gene plays an important role in viral replication and virulence (Johnson, 2010), the T antigen-like region is considered to be involved in virulence. We previously detected JEECV in the Japanese eels (all in the yellow stage) caught in freshwater and brackish water sites of two river systems in Japan, and in Japanese elvers (glass eel stage) caught in the Asagawa river in Japan. In that study, single base substitution was confirmed among the sequences of the T antigen-like gene in JEECV obtained from the yellow-staged eels, and two-base substitution was confirmed among them obtained from the yellow-staged eels and farmed eels (Okazaki *et al*., 2015; Okazaki *et al*., 2016). These findings indicated that habitat-wide epidemiological studies with different life stage of eels are necessary to study the viral strains and to clarify the infection mechanism of JEECV.

By finding newly hatched pre-leptocephali of Japanese eels, the spawning area of the Japanese eel was determined to be located in the southern part of the West Mariana Ridge in the North Pacific (Tsukamoto, 2006). More recently, four mature Japanese eel (two males and two females) were captured for the first time in the putative spawning area in 2008, using a large midwater trawl (Chow *et al*., 2009; Kurogi *et al*., 2011). To date, a total of 15 Japanese eels were caught in four research cruises (Tsukamoto *et al*., 2011; unpublished information from Dr. H. Kurogi), and some of these individuals became available to study JEECV infection in mature Japanese eels collected in their spawning area.

To detect JEECV, we used the quantitative PCR (qPCR) assay developed by Mizutani *et al*. (2011), and for further confirmation, a conventional PCR was performed as described previously (Mizutani *et al*., 2011). Since JEECV was found in the nuclei of endothelial cells in the gills, liver and kidney using electron microscopy (Inouye *et al*., 1994; Mizutani *et al*., 2011), we selected these three as the target organs for JEECV detection.

Five mature wild eels caught in the southern part of West Mariana Ridge in 2013 were used in this study.

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Four were female (no. 1, 2, 3 and 5) and one was male (no. 4). The eels had no apparent abnormalities. Gills, liver and kidney were dissected on board immediately after the catch, and stored at –80°C until use. The samples were homogenized using a sterilized biomasher II (Nippi), and viral total DNA was extracted using the High Pure Viral Nucleic Acid Kit (Roche), to which the above mentioned detection methods were applied. Briefly, a qPCR was performed with TaqMan Gene Expression Master Mix in a 7300 Real Time PCR System (Applied Biosystems). The minimum detection limit of qPCR was the 100 copies/reaction. The PCR was performed with AmpliTaq Gold PCR Master Mix (Applied Biosystems) using primer set B (Mizutani *et al*., 2011). The expected size of PCR product was 250 bp. The PCR products were electrophoresed on 1.5% agarose gel followed by staining with ethidium bromide. In this study, we diagnosed JEECV-positive by the detection of the JEECV genome in both qPCR and the PCR, which were performed twice.

To sequence a part of the genome of the large T antigen-like gene, nested PCR was performed using the samples in which JEECV was detected by both qPCR and the PCR. Nested PCR was conducted with Premix Ex Taq Hot Start Version (Takara) using primer set E (Okazaki *et al*., 2015). The amplified products were electrophoresed on 1.5% agarose gel and purified using the MonoFas DNA Isolation system (GL Science). The purified DNA was sequenced bi-directionally with the Big Dye Terminator ver. 3.1 Cycle Sequence Kit (Applied Biosystems) using an ABI 3130 Genetic Analyzer (Applied Biosystems). Sequences obtained were analyzed using a Sequence Scanner ver. 1.0 (Applied Biosystems) and BLAST (National Center for Biotechnology Information).

Table 1 shows that JEECV genome was detected in the gills from eel no. 1 and 4 by the qPCR (left column: gene copies per reaction; right column: gene copies per mg tissue). One of the reasons that JEECV in no. 4 was not detected at the second test is thought to be degradation of viral DNA by freeze-thaw after the first test. Therefore, no.1 was used in future study. Fig. 1 shows the result of electrophoresis of PCR product of no.1. The sequencing of a part of the PCR products showed that the sequences of JEECV DNA detected from the gills of eel no. 1 were identical to those of JEECV isolated from Japanese eels inhabiting freshwater areas (GenBank accession no. AB920751). The nucleotides of C (at the position 14,123) and A (at the position 14,194) of JEECV isolated from farmed eels with VECNE were changed to G of the no. 1 eel. From the result, amino acid at the position 218 (nucleotide change: A to G at the position 14,194) of the predicted polyomavirus large T like protein should be changed from threonine to alanine. In the qPCR assay in this study, JEECV was not detected in the liver and kidney samples (data not shown). Since the T antigen gene plays an important role in viral replication and virulence in human polyomaviruses (Johnson, 2010), further studies with epidemiological investigation and experiment using isolated JEECV are necessary to clarify relationship between the pathogenicity and the amino acid change.

This study is the first report of JEECV infection of mature adult Japanese eels caught in their spawning area; however, it should be noted that JEECV was detected only in one of the five mature Japanese eels used in our study. Previously, JEECV infection has been detected in the Japanese eels at glass eel stage

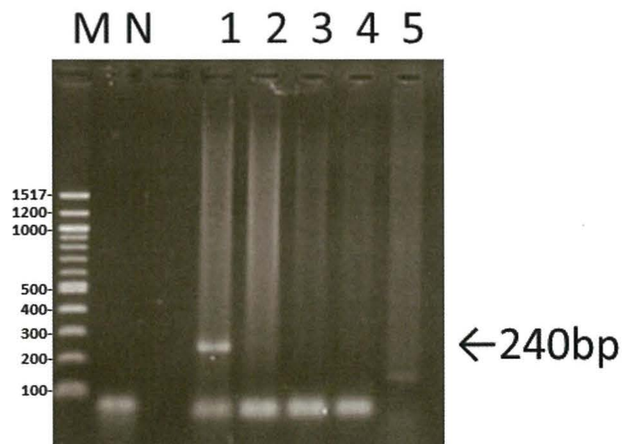

Fig. 1. Gel electrophoresis of JEECV specific product amplified by PCR using primer set B from the gills of mature Japanese eel collected in the spawning area. Lane 1–5, ID of samples; M, DNA marker (100 bp ladder); N, Negative control (Nuclease-free water).

Table 1. Detection of JEECV in specimens of mature Japanese eels (*Anguilla japonica*)

sample ID	gene copies/reaction of tissue			gene copies/mg of tissue		
	Gill (1st[a]/2nd[b])	Liver	Kidney	Gil (1st[a]/2nd[b])	Liver	Kidney
1 ♀	310/118	Neg[c]	Neg	1568/490	Neg	Neg
2 ♀	Neg	Neg	Neg	Neg	Neg	Neg
3 ♀	Neg	Neg	Neg	Neg	Neg	Neg
4 ♂	121/Neg	Neg	Neg	694/Neg	Neg	Neg
5 ♀	Neg	Neg	Neg	Neg	Neg	Neg

a: The sample whose DNA was extracted on Februrary 14, 2014
b: The sample whose DNA was extracted on September 16, 2014
c: Negative

and yellow stage caught in water systems in Japan (Okazaki *et al.*, 2015; Okazaki *et al.*, 2016). It is likely that JEECV infects to Japanese eels living in natural environments and it will be necessary to detect JEECV in their gonadal tissues. These findings would further contribute in clarifying the manner of JEECV infection in Japanese eels living in natural habitats. Recently, some researchers have shown interest in the severe pathogenic agents, including parasites, viruses and bacteria that have potentially adverse effects on spawning migration (ICES, 2006). Further studies should be conducted to define the effects of JEECV infection on the spawning migration of Japanese eels.

Acknowledgements

We would like to express our gratitude to the captain and crews of R/V Kaiyo Maru, and to the Fishery Agency of Japan for their help in the sample collection. We are grateful to the Fishery Agency of Japan and the Fisheries Research Agency for their support.

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天然ニホンウナギ親魚からの Japanese eel endothelial cells-infecting virus（JEECV）の検出

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JEECVは，鰻養殖で被害が大きいウイルス性血管内皮壊死症の原因である。我々は日本近海のニホンウナギにJEECVが感染していることを明らかにしたが，産卵場の親魚では不明である。そこで，本研究では2013年に西マリアナ海嶺南部海域の産卵場で採捕した親魚5尾の鰓，肝臓および腎臓を対象に分子生物学的手法を用いてJEECV感染を調査した。その結果，雌1尾の鰓からJEECVの polyomavirus large T like protein 遺伝子が検出された。その推定アミノ酸配列は河口や汽水域に生息するニホンウナギから検出されたJEECVと同一であったが，養殖場で分離された株とは1アミノ酸異なっていた。今回の結果は産卵場の親魚の病原体保有状況を明らかにした初めての報告である。

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