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NOTE

Amoebic gill infection in coho salmon *Oncorhynchus kisutch* farmed in Korea

Wi-Sik Kim, Kyoung-Hui Kong, Jong-Oh Kim, Myung-Joo Oh*

Department of Aqualife Medicine, College of Fisheries and Ocean Science, Chonnam National University, Yeosu 59626, South Korea

ABSTRACT: About 70% mortality occurred in cultured coho salmon *Oncorhynchus kisutch* at a marine farm in the South Sea of Korea in 2014. Diseased fish showed greyish or pale patches on the gills, with no internal signs of disease. No bacteria or viruses were isolated from diseased fish, but numerous amoebae were found on the gills. Histopathological examinations revealed extensive hyperplastic epithelium and lamellar fusion in the gills. Numerous amoebae were seen between gill filaments. The amoebae had a 630 bp partial 18S rRNA gene fragment specific to *Neoparamoeba perurans*. Phylogenetic analysis based on partial 18S rRNA gene nucleotide sequences revealed that this Korean amoeba belonged to the *N. perurans* group. This is the first report of *N. perurans* infection in Korea.

KEY WORDS: Amoebic gill disease · AGD · Coho salmon · *Neoparamoeba perurans*

INTRODUCTION

Amoebic gill disease (AGD) is a serious parasitic disease affecting salmonids cultured in the marine environment in Tasmania (Australia), North America, and Europe (Munday et al. 2001, Mitchell & Rodger 2011, Oldham et al. 2016). *Neoparamoeba perurans*, *N. pemaquidensis*, and *N. branchiphila* have been isolated or detected from diseased fish (Mitchell & Rodger 2011, Oldham et al. 2016). Of these amoebae, *N. perurans* has been shown to be the causative agent of AGD worldwide (Young et al. 2008a, Crosbie et al. 2012). AGD is most commonly reported at temperatures between 12 and 20°C with salinities approaching 35‰ (Munday et al. 2001, Mitchell & Rodger 2011). Affected fish show multifocal patches or spots of white to grey swollen gill tissues with excess mucus surrounding the gill arches (Rodger & McArdle 1996, Mitchell & Rodger 2011). The main histological feature of AGD is prominent epithelial hyperplasia in gill tissues, resulting in complete lamellar fusion (Kent et al. 1988, Mitchell & Rodger 2011).

In Far East Asia, amoebic gill infection was reported in cultured olive flounder *Paralichthys olivaceus* in 2005 in Korea (Kim et al. 2005) and ayu *Plecoglossus altivelis* in 2008 in Japan (Crosbie et al. 2010). However, amoebae and amoebic infection have not been reported in olive flounder, ayu, or other marine fish after that time. In 2014, a high mortality of about 70% occurred in cultured coho salmon *Oncorhynchus kisutch*. Amoebae were detected in diseased fish after bacteriological, parasitological, and virological examinations. In this study, we report an amoebic infection of cultured coho salmon in Korea.

*Corresponding author: ohmj@jnu.ac.kr

MATERIALS AND METHODS

Coho salmon

In total, 3000 coho salmon (weighing 120–400 g) were moved to a marine farm (land-based system) located in Jeju-do from a freshwater aquaculture farm in Kangwon-do in 2014. These fish were first maintained in underground freshwater at 14–15°C for 7 d before adapting to seawater at 5‰ per week using a method of gradual transfer to seawater. After seawater acclimation (33‰), fish were reared in square tanks (6 × 6 × 1 m) using coastal and underground seawater. Water temperatures of 15 to 17°C were maintained by mixing seawater from the 2 sources.

Examination of diseased coho salmon

Moribund fish were removed from the coho salmon population at the Jeju farm. Five moribund fish (169–367 g) were collected for disease examination. Fish were transported on ice and immediately subjected to parasitological, bacteriological, and virological examinations. The gills and body surfaces were microscopically examined for parasites. Pieces of the kidney, spleen, and liver were cultured in brain heart infusion agar plates (Difco) supplemented with 1% NaCl and incubated at 15°C for 14 d to isolate the bacteria. The kidney and spleen samples were homogenized in 10 volumes of Hanks' balanced salt solution (HBSS, Gibco) and centrifuged at 3000 × *g* (20 min). The supernatant was filtered through a 450 nm membrane filter, and 100 µl of the homogenate were inoculated onto Chinook salmon embryo (CHSE-214) and fathead minnow (FHM) cells in 24-well tissue culture plates (Nunc). The inoculated cells were then incubated at 15°C for 14 d and examined daily for cytopathic effect. For histology work, gills were removed from diseased fish and immediately fixed in 10% neutral buffered formalin. After fixation, standard histological procedures were used for tissue dehydration and paraffin embedding. Tissue sections were stained with hematoxylin and eosin (H&E).

Polymerase chain reaction (PCR) and sequencing analyses

Gill swabs of diseased fish were used for PCR analysis. Amoebic genomic DNA was isolated using a Genomic DNA Extraction Kit (Bioneer) following the manufacturer's instructions. DNA templates were subjected to a PCR assay with forward (5′-ATC TTG ACY GGT TCT TTC GRG A-3′) and reverse (5′-ATA GGT CTG CTT ATC ACT YAT TCT-3′) primers to amplify a 636 bp region of the *Neoparamoeba perurans* 18S rRNA gene (Young et al. 2008b). PCR was carried out in a thermal cycler (MyGenie 96 thermal block, Bioneer) using PCR premix (Bioneer) following the manufacturer's instructions. PCR cycle conditions were 94°C for 5 min; 35 cycles of 94°C for 30 s, 50°C for 30 s, and 72°C for 45 s; and 72°C for 5 min. PCR products were subjected to 1.5% agarose gel electrophoresis and purified using a QIA quick Gel Extraction Kit (Qiagen). Purified PCR product was cloned into pCR 2.1-Topo vector system (Invitrogen) and transformed to *Escherichia coli* strain TOP10 (Invitrogen). Sequencing was performed using an ABI PRISM 3730 XL DNA analyzer (Applied Biosystems) with M13 reverse and T7 promoter primer set. The resulting sequences were assembled with the MEGA6 program (Tamura et al. 2013). The 588 bp sequences (isolate: CSJeju14) without primer sequences were submitted to GenBank (accession number KU985055) and compared to those of 47 worldwide amoebae (Table 1). Multiple sequence alignment was conducted using Clustal X (Thompson et al. 1997) to infer the genetic relationships among sequences with a neighbor-joining algorithm. The final phylogenetic tree was drawn with MEGA6.

RESULTS AND DISCUSSION

In 2014, mortality of coho salmon was first observed at 60 d after seawater acclimation (33‰). Fish death continued for 2 mo, ultimately reaching a cumulative mortality of approximately 70%. Affected fish were lethargic and unresponsive to stimuli. Diseased fish exhibited greyish gills (2 of 5 fish) or pale patches on the gills (3 of 5 fish), without internal signs of disease (Fig. 1A). Upon examination, 100% of gills of diseased fish were infected with numerous amoebae (Fig. 1B). No other parasites except amoebae were found on the gills or the body surface of diseased fish. No bacteria and viruses were isolated from diseased fish. Histopathological examinations (2 fish) revealed extensive hyperplastic epithelium and lamellar fusion in the gills (Fig. 1C). Numerous amoebae were seen between gill filaments (Fig. 1C,D). Clinical and histological signs of diseased fish were similar to those of AGD reported pre-

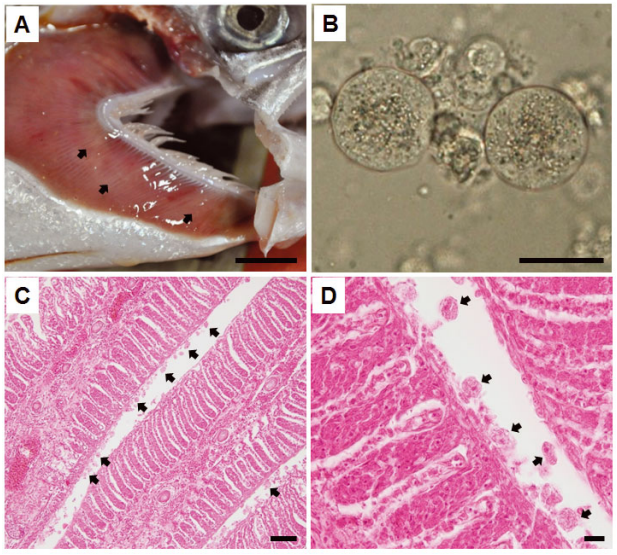

Fig. 1. Amoebic gill infection in cultured coho salmon *Oncorhynchus kisutch*. (A) Diseased fish showing slightly pale patches (arrows) on the gills. (B) Amoebae found on the gills. (C,D) Histopathological examination revealed extensive hyperplastic epithelium and lamellar fusion in the gills and numerous amoebae (arrows) around these lesions. Scale bars = (A) 1 cm, (B) 25 µm, (C) 100 µm, (D) 20 µm

viously (Kent et al. 1988, Mitchell & Rodger 2011). These results confirmed that severe amoebic infection occurred in diseased coho salmon.

The identity of the amoebae was determined using PCR and sequencing analyses. The amoebae showed a 630 bp DNA fragment specific for *Neoparamoeba perurans* (data not shown). When PCR products were sequenced, the nucleotide sequences of the 588 bp fragment (except primer sequences) showed 99.8% homology with the *N. perurans* LB200313-1 isolate (KF179520) from ballan wrasse *Labrus bergylta* in Norway (data not shown). A phylogenetic tree based on the 18S rRNA gene of 48 worldwide amoebae revealed that the Korean amoeba (CSJeju14) belonged to the *N. perurans* group (Fig. 2, Table 1). These results confirmed that the Korean amoeba was *N. perurans*.

In Korea, amoebic (*Neoparamoeba* sp.) infection in marine fish was first reported in cultured juvenile olive flounder in 2005 (Kim et al. 2005); thereafter, no amoebic infection was reported in any marine fish. In the present study, *N. perurans* infection

Table 1. GenBank accession numbers for amoebae isolates shown in Fig. 2. The isolate from the current study is indicated in **bold**

Species and strain	GenBank acc. no.	Species and strain	GenBank acc. no.
Neoparamoeba pemaquidensis		*Neoparamoeba perurans*	
GILLNOR1	AY714352	GD-D1/1/2	EF216903
NP251002	AY714351	GD-D1/3	EF216900
GILLRICH3/I	EF675606	GD-D1/2	EF216899
NET12AFL/I	EF675604	GD-HAC/2/1	EF216904
NETH2T3	AY714350	**CSJeju14**	**KU985055**
TUN1/I	EF675607	Neo-D261006	EU326494
FRS	AY714356	LB200313-1	KF179520
ST8V	AY714355	GD-HAC/2/2	EF216905
CCAP1560/4	AF371969	GD-D1/1/1	EF216902
CCAP1560/5	AF371970	GD-D1/4	EF216901
AFSM2V	AY193722	*Neoparamoeba branchiphia*	
AFSM11	AY193723	AMOPI	EF675600
SEDCB1	AY714357	NRSS	AY714367
PA027	AY714358	ST4N	AY714365
NETC1	AY714363	SM68	AY193725
WT2708/I	EF675605	TG1162	EF675601
ATCC30735	AF371972	SEDMH1	AY714366
ATCC30735	AY183887	RP	EF675603
Neoparamoeba aestuarina		TG1267	EF675602
SU03	EU331035	SU4	EF675599
CCAP1560/7	AY686574	AFSM3	AY193724
W4-3	DQ229957	SM53	AY193726
SL200	DQ229959	*Paramoeba eilhardi*	
S131-2	DQ229958	CCAP1560/2	AY686575
ATCC50806	AY121852		
ATCC50805	AY121851		
ATCC50744	AY121848		

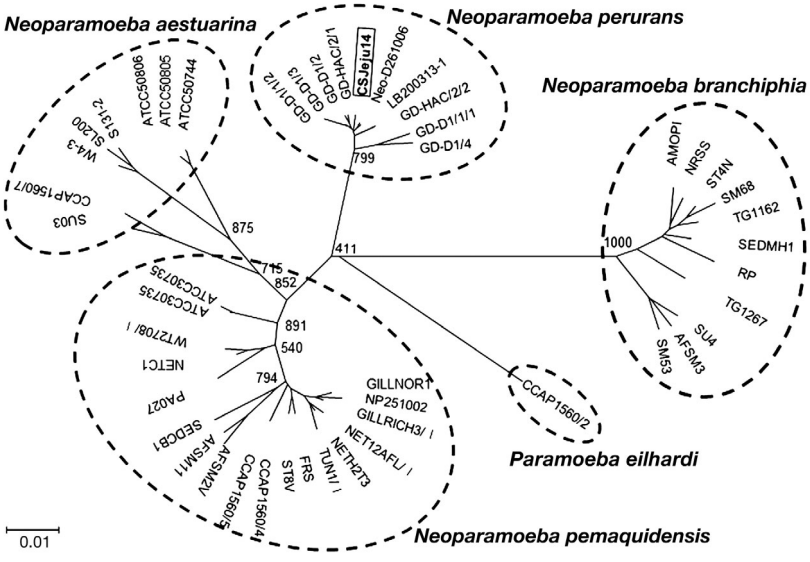

Fig. 2. Molecular phylogenetic tree showing the genetic relationships among 48 worldwide amoebae based on partial 18S rRNA gene nucleotide sequences (see Table 1 for GenBank accession numbers). Bootstrap values for 1000 replicates are shown at major tree nodes. The distance marker refers to the expected number of substitutions per site. The isolate from the current study is indicated in **bold**

was confirmed in coho salmon from an aquaculture farm in Jeju in 2014. This is the first study to report *N. perurans* infection in cultured coho salmon in Korea, even though coho salmon are known to be susceptible to AGD in the USA and Chile (Kent et al. 1988, Rozas et al. 2012). It remains unclear why the amoebic infection was not reported until 2013 after the first outbreak and whether amoebae affect marine fish of Korea. Therefore, continuous surveys and surveillance of amoebae will be performed in future studies.

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