Occurrence of Bacterial Kidney Disease in Cultured Ayu

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ABSTRACT—A disease characterized by white nodules in the kidney, swollen abdomen and ascitic fluid occurred in cultured ayu Plecoglossus altivelis from April to July, 2001. The bacterial isolate on KDM-2 from the affected ayu was identified to Renibacterium salmoninarum using IFAT and PCR, and experimental infection resulted in the same clinical signs and re-isolation of the bacterium. From clinical signs, bacteriological examinations and challenge test, this disease in ayu was diagnosed as bacterial kidney disease (BKD). In experimental infection, cumulative mortalities of ayu and masu salmon Oncorhynchus masou were 100% and 60% when injected with 10^7 cells/fish, and 100% and 0% when injected with 10^3 cells/fish, respectively. These results indicate that ayu was more susceptible to the isolate than masu salmon, although there is no reported case of BKD in ayu. The affected ayu were originally introduced from another farm in April 2001, where salmonid fishes were reared in neighboring ponds, and it was revealed that masu salmon cultured in the latter farm was infected with R. salmoninarum. This result indicates that R. salmoninarum was horizontally transmitted from masu salmon to ayu.

Key words: bacterial kidney disease, Renibacterium salmoninarum, Plecoglossus altivelis, BKD, ayu

Bacterial kidney disease (BKD), caused by Renibacterium salmoninarum, is one of the most important diseases of wild and cultured salmonid fishes in many countries. R. salmoninarum can be transmitted vertically and horizontally, and causes a systemic and usually chronic disease, resulting in a significant economic loss (Evelyn, 1993; Evenden et al., 1993). Natural outbreaks of BKD have been reported only in salmonids (Wiens and Kaattari, 1999; Yoshimizu, 2000). In Japan, this disease was first reported from chinook salmon Oncorhynchus tschawytscha, sockeye salmon O. nerka, pink salmon O. gorbuscha and masu salmon O. masou (Kimura and Awakura, 1977) in Hokkaido and rapidly spread to other parts of the country.

From April to July in 2001, a disease resulting in mass mortality occurred in cultured ayu Plecoglossus altivelis in a farm in Hiroshima Prefecture, Japan. The clinical signs similar to those of BKD in salmonid fish, i.e. white nodules in the kidney, swollen abdomen and ascitic fluid, were observed in affected fish. From the results of bacteriological examinations and challenge tests as well as the clinical signs, this disease of ayu was diagnosed as BKD. This is the first reported case of BKD in cultured ayu. Furthermore, the effect of water temperature on the pathogenicity of the isolate and the origin of R. salmoninarum in ayu was investigated.

Materials and Methods

Fish examined and bacterial isolation
Moribund ayu (16.9 to 39.0 g in body weight) were sampled from the private farm (farm A) where the disease was occurring in July. After external and internal observations of the samples, bacterial isolation was performed from the kidney using tryptosoya agar (TSA, Nissui) and KDM-2, which were incubated at 18°C. In addition, kidney smears were examined with indirect fluorescent antibody technique (IFAT) using an anti-R. salmoninarum (ATCC33209 strain) rabbit serum.

Bacteriological examination
Bacteria isolated on KDM-2 from the kidney were examined to be R. salmoninarum or not by IFAT and polymerase chain reaction (PCR). Isolated bacteria were also examined to grow on KDM-C (Daly and Stevenson, 1985) or KDM-2 without serum. PCR was performed using a primer set designed to amplify a 501 bp DNA segment of the gene encoding the p57 protein produced by R. salmoninarum (Brown et al., 1994; 1995). For PCR, cultured bacteria or the kidney of affected ayu were used as templates.

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fected fish were homogenized with sterile distilled water containing tween 20 (0.5%) and proteinase K (0.5 mg/mL) and incubated for 15 min at 37°C, and the total nucleic acids were extracted with phenol saturated TE buffer (10 mM Tris-HCl pH 8.0, 1 mM EDTA pH 8.0) and chloroform-isoamyl alcohol (24:1). PCR amplification was carried out under the same condition described by Brown et al. (1994). Type strain of R. salmoninarum, ATCC33209 (Sanders and Fryer, 1980), was used as the positive control.

Experimental infection

Apparently healthy ayu (average weight, 35 g) and masu salmon (22 g) were used for infection experiments. These fish were reared in UV-treated fresh well or dechlorinated tap water at Hiroshima Prefectural Fisheries Experiment Station, and no disease signs including BKD were observed. A representative bacterial strain, PH-0110 isolated from a diseased ayu, was grown on KDM-2 for 10 days at 18°C and suspended in PBS (–) at 1 mg/mL (10⁶ cells/mL). The number of cells was counted under a light microscope with a bacteria counter. Fish anaesthetized by FA-100 (4-allyl-2-methoxyphenol, Tanabe Seiyaku) were injected intraperitoneally with the isolate at 10⁷ or 10⁵ cells/fish. The control fish received injections of an equivalent volume (0.1 mL) of sterile PBS (–). Fifteen ayu and 30 masu salmon of each group were used in this experiment. Water temperature was maintained at 18°C during the experiment.

To examine the effect of water temperature on the disease progress in ayu, 10 fish of each group were injected intraperitoneally with 10⁷ cells/fish and maintained at 18°C or 25°C.

In each experiment kidney smear specimens of dead fish were submitted to IFAT to detect R. salmoninarum antigen, and bacterial re-isolation was attempted from the kidney of some dead fish on KDM-2. At the termination of experiments, the kidney of all surviving fish was submitted to PCR to detect R. salmoninarum gene as describe above.

Survey of the source of R. salmoninarum in ayu

A survey on the source of the infection was conducted in a farm (farm B) in October 2001, from which ayu were introduced into the farm A in April. In the farm B ayu and salmonid fish were reared in different ponds from January to April 2001. Six female rainbow trout O. mykiss (average weight, 1,003 g) and 21 female masu salmon (168 g) were sampled (rearing water temperature at sampling was 13.4°C), and each fish was examined for internal and external abnormalities. The kidney, ovarian fluid and one egg from each fish was examined to detect R. salmoninarum gene by PCR. The ovarian fluid was centrifuged at 10,000 x g for 10 min, and obtained deposit was used for PCR. Bacterial isolation was performed using KDM-2 from the fish exhibiting white nodules in the kidney, and isolated bacteria was identified as R. salmoninarum by PCR.

Results

Disease occurrence and bacterial isolation

Mortality of ayu was chronic from April to July and a cumulative loss was more than 50%. The water temperature during the epizootic ranged between 15 and 21°C. Seven moribund fish were examined and all fish showed swollen abdomen and exophthalmos externally, and internally accumulated clear ascitic fluid and white nodules in the kidney, moreover two fish showed white nodules in the liver (Fig. 1). Small gram-positive bacilli
were found in the smear specimens of the kidney of all examined fish (Fig. 2), and the bacteria positively reacted with anti-\textit{R. salmoninarum} serum by IFAT. No dominant bacteria grew on TSA, but dominant colonies of a bacterium were isolated from the kidney of all examined fish on KDM-2 incubated for 14 days at 18°C. A representative isolate, PH-0110, grew on KDM-C and KDM-2 without serum as well as KDM-2. The PCR product approximately 500 bp was amplified from the genome of PH-0110 as well as the type strain (Fig. 3). The same PCR product was amplified from nucleic acids extracted from the kidney of affected fish.

\textbf{Infection experiments}

Cumulative mortality of ayu and masu salmon injected with PH-0110 is shown in Fig. 4. Ayu injected with $10^7$ and $10^3$ cells of PH-0110 began to die at 15 and 28 days post-injection, and the cumulative mortality reached 100\% within 17 and 52 days, respectively. Masu salmon inoculated with $10^7$ cells of PH-0110 began to die at 18 days post-injection, and the cumulative mortality reached 60\% after 61 days, while no mortality was observed in $10^3$ cell-challenged masu salmon during the experiment.

The dead ayu and masu salmon showed the same clinical signs, i.e. swollen abdomen, exophthalmos and accumulated clear ascitic fluid. However, the white nodules were rarely observed in the kidney and liver. \textit{R. salmoninarum} antigens were detected with IFAT from kidney smears of each dead fish, and the same bacterium was re-isolated from the kidney of dead fish. No \textit{R. salmoninarum} gene was detected by PCR from the kidney of surviving fish, except for one masu salmon inoculated with $10^7$ cells among 12 survivors.

\textbf{Source of \textit{R. salmoninarum}}

No white nodules were observed in the kidney of rainbow trout, but the lesions were observed in the kidney of one masu salmon (No.4 fish) sampled from the farm B. The lesions were filled with gram-positive bacteria showing positive reaction with the IFAT. A bacterial strain (OH-0114) isolated form the kidney of No.4 fish was confirmed as \textit{R. salmoninarum} using IFAT and PCR. \textit{R. salmoninarum} genes were not detected from all rainbow trout, but were detected from the kidney (1/21, positive/examined fish), ovarian fluid deposit (4/19).
Table 1. Detection of *Renibacterium salmoninarum* by PCR from rainbow trout and masu salmon cultured in the farm B from which ayu derived

<table>
<thead>
<tr>
<th>Species</th>
<th>Fish No.</th>
<th>PCR</th>
<th>PCR</th>
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<tbody>
<tr>
<td></td>
<td></td>
<td>Kidney</td>
<td>Ovarian fluid</td>
</tr>
<tr>
<td>Rainbow trout</td>
<td>1-4</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td><em>(n=6)</em></td>
<td>5, 6</td>
<td>NT*</td>
<td>–</td>
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<tr>
<td></td>
<td>1</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>Masu salmon</td>
<td>3</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td><em>(n=21)</em></td>
<td>4</td>
<td>+**</td>
<td>+</td>
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<tr>
<td></td>
<td>5-19</td>
<td>–</td>
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<tr>
<td></td>
<td>20, 21</td>
<td>NT</td>
<td>–</td>
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*NT: not tested
**The white nodules were observed in the kidney.

and eggs (3/21) of masu salmon using PCR (Table 1).

Discussion

In the present study, the etiology of the disease with a mass mortality in cultured ayu was examined. The clinical signs of affected ayu, swollen abdomen and exophthalmos, accumulated ascitic fluid and white nodules in the kidney and liver, were similar to those of BKD in salmonid fish. Especially the white nodules regarded as the pathognomonic of BKD were recognized commonly in the kidney of affected fish. Furthermore these lesions showed positive reaction in IFAT with the anti-*R. salmoninarum* serum. The representative isolate PH-0110 reacted positively with the antiserum in IFAT and with the primer set specific to *R. salmoninarum* in PCR amplification, and reproduced the disease conditions in ayu by the intraperitoneal challenge. From these results PH-0110 was identified as *R. salmoninarum* and the disease was diagnosed as BKD.

*R. salmoninarum* infections have been produced experimentally in five species of non-salmonid fish, Pacific herring *Clupea harengus pallasi*, sablefish *Anoplopoma fimbria*, shiner perch *Cymatogaster aggregata*, common shiner *Notropis cornutus* and flathead minnow *Pimephales promelas*, however, documented reports of BKD in non-salmonids, commercially important or otherwise, do not exit (Evelyn, 1993). Therefore this is the first report of natural outbreak of BKD in non-salmonid fish, although it is impractical in salmonid fish.

A survey on the source of BKD in ayu was carried out in the farm B, because the ayu, later became BKD, were introduced from this farm where salmonid fishes were also reared. It was revealed that one of masu salmon showed the clinical signs of BKD, and *R. salmoninarum* genes were detected from some of masu salmon in the farm B. Additionally, OH-0114 strain identified as *R. salmoninarum* was isolated from the kidney of masu salmon there. The occurrences of BKD in salmonid fishes have not been confirmed at the farm B. In the farm B ayu were reared in a neighboring pond of masu salmon before they were transferred to the farm A. Balfry *et al.* (1996) reported that the fecal-oral route of horizontal transmission might contribute significantly to increase prevalence of BKD in farmed salmon. It seems likely that *R. salmoninarum* was transmitted from masu salmon to ayu horizontally in the farm B when they were cultured closely together. It is necessary to compare genetically *R. salmoninarum* PH-0110 from ayu with OH-0114 from masu salmon in detail as well as in vivo transmission experiment to make sure that *R. salmoninarum* in salmonid fish was transmitted to ayu.

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