多摩川支流域で認められたアユ死亡に関与する病原性
Aeromonas veronii biovar sobria

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Short communication

Aeromonas veronii biovar sobria
Associated with Mortalities of Riverine Ayu Plecoglossus altivelis in the Tama River

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ABSTRACT—In July 2016, there were mortalities of riverine ayu Plecoglossus altivelis in a tributary of the Tama River, Japan. A Gram-negative, motile and short rod-shaped bacterium was dominantly isolated from all examined dead fish, and identified as Aeromonas veronii biovar sobria. Biochemical characteristics and gyrB sequence of the present strains differed from those of A. veronii strains from ayu in previous years. The present strains also caused higher mortalities to ayu than A. veronii strains previously isolated. These results indicate that the present mortalities of riverine ayu in the Tama River were caused by high pathogenic A. veronii biovar sobria.

Key words: Aeromonas veronii biovar sobria, Plecoglossus altivelis, riverine fish, pathogenicity, the Tama River

The ayu Plecoglossus altivelis, a representative freshwater fish species in Japan, has long held an important position among riverine fishes as a target for recreational fisheries and as food for human consumption. Therefore, hatchery-produced or wild (captured from lakes, rivers or sea coasts) ayu are released annually into many rivers to enhance riverine stocks, despite indications that many released fish are at risk of several bacterial infections. In particular, bacterial infection by Flavobacterium psychrophilum (bacterial cold-water disease) (Iida and Mizokami, 1996) and Edwardsiella ictaluri (Sakai et al., 2008) has become one of the most serious problems for riverine ayu management in recent years. Consequently, the infection status of both pathogens in hatchery-produced and wild ayu has been investigated throughout Japan (Kumagai, 2016; Iida et al., 2016).

In July 2016, bacteria that differed from F. psychrophilum and E. ictaluri were isolated from dead ayu collected following mass mortalities of riverine ayu in the tributary of the Tama River, Japan. The bacteria were identified as Aeromonas veronii, which has been frequently isolated from aquatic environments (Albert et al., 2000) and fish intestines (Namba et al., 2007). Although some studies report that A. veronii causes disease in farmed and ornamental fishes (Rahman et al., 2002; Sreedharan et al., 2011; Smyrli et al., 2017), the majority of aeromonads causing damage in Japanese aquaculture have been identified as A. hydrophila and A. salmonicida (Jo and Onishi, 1980; Kitao et al., 1985; Rahman et al., 2001; Yamamoto, 2017). In the present study, we investigated the characteristics and pathogenicity of strains from diseased ayu and concluded that the mortalities of riverine ayu found in the tributary were caused by A. veronii.

Materials and Methods

Bacterial examination

There were two mass mortalities of riverine ayu in the tributary of the Tama River in July 2016, when the daily average water temperature rapidly increased above 23°C. Since we could not sample freshly dead ayu in the first mortality event, we obtained 16 dead and 14 living fish (captured by angling) in the second event. Bacterial isolation from the kidney were performed using trypto-soya agar (TSA, Nissui) and the plates with inoculum were incubated for 48 h at 25°C. Cell morphology and motility of the bacterial strains were examined microscopically by Gram staining and the wet-mount method, respectively, and strains were molecularly identified to species using a partial (500-bp) 16S rRNA sequence from the 5′ region (Namba et al., 2007). Additionally, we tested for the presence of F. psychrophilum and E. ictaluri in sampled ayu according to the methods of our previous study (Takeuchi et al., 2016).

Biochemical and phylogenetic characterization

Of the strains identified as A. veronii in the present study by partial 16S rRNA sequencing, 15 strains ("present strains") were biochemically characterized using API 20E (BioMerieux) according to the manufacturer's instructions. The derived API profiles were compared with those of the eight A. veronii strains from the kidney of ayu and pale chub Opsarichthys platypus captured in the Tama River Basin in 2012 and 2014 ("previous strains" from asymptomatic or E. ictaluri infected fish), and reference strains from the intestine of common carp.
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Cyprinus carpio (HPI4 and CWP11; Namba et al., 2008) and human patients (JCM7375; Hickman-Brenner et al., 1987). Additionally, for phylogenetic characterization, we performed gyrB gene (1,100 bp) amplification and direct sequencing using extracted DNA from present strains and the primers developed by Yanez et al. (2003). After genome assembly, the sequences were compared to sequence data in GenBank using BLAST (blastn) algorithms (https://blast.ncbi.nlm.nih.gov/Blast.cgi), and aligned with sequences of previous strains and reference strains using Clustal X (Thompson et al., 1997). A maximum likelihood phylogenetic tree of aligned sequences was constructed with Kimura’s 3-parameter model using MEGA 6 software (http://www.megasoftware.net/), and the robustness of the phylogenetic results were tested by bootstrap analysis with 1,000 iterations.

Experimental infection

To assess the pathogenicity of A. veronii strains obtained in the present study, we performed an experimental infection of ayu using the three present strains from dead ayu (AAr1608, AAr1614, and AAr1615), three previous strains (AAr1412, AAr1216, and AAr1218), and one reference strain from the intestine of common carp (CWP11). Stock cultures of all strains in tryptic soy broth (TSB, Difco) containing 10% glycerol at −80°C, were transferred and grown on TSA at 25°C for 24 h and then cultured in 300 ml TSB with shaking at 25°C for 9 h. Hatchery-produced ayu (body weight: 12.0 ± 1.9 g), obtained from the Freshwater Experimental Station, Kanagawa Prefectural Fisheries Technology Center, were acclimated to experimental conditions at 20°C–25°C for 5 days prior to the experiment.

Following a 10-fold dilution of bacterial suspensions of each strain with dechlorinated tap water (final bacterial density: 2.1–3.4 × 10⁷ CFU/mL), experimental fish were immersed in the suspensions at 25°C for 30 min, while control fish were exposed to 10-fold diluted TSB. The fish exposed to each strain (n = 10 per strain) were then reared at 25°C in a 50-L glass aquarium with filtration and aeration equipment, and monitored for 10 days. Identification of isolates from the kidneys of dead and moribund fish was performed by direct sequencing of the gyrB gene as described above.

Results and Discussion

Most dead ayu and some living fish obtained during the mass mortality of riverine ayu in the river tributary showed external and internal clinical signs such as hemorrhaging of the lower jaw or body surface, reddening at the base of the ventral fin or anus, and ascites (Fig. 1). Of these signs, reddening of the anus and ascites are known as typical clinical signs of E. ictaluri infection (Sakai et al., 2008; Takeuchi et al., 2016), so at first, we assumed that the mortality was caused by E. ictaluri. However, E. ictaluri was detected in only 31.3% (five of 16 fish) of dead ayu and 28.6% (4/14) of the living; F. psychrophilum was not detected in any sampled fish. On the other hand, unknown bacteria, which were Gram negative, motile, and short rod-shaped, were isolated from 100% (16/16) of dead ayu and 57.1% (8/14) of living fish. The partial 16S rRNA sequences derived from these bacteria (accession nos., LC311422- LC311447) most closely matched that of A. veronii (KT998815).

A. veronii was originally described as a novel species in the genus Aeromonas in 1987 (Hickman-Brenner et al., 1987). It is divided into two biovars ("sobria" and "veronii") on the basis of biochemical characteristics such as the activity of arginine dihydrolase (ADH) and ornithine decarboxylase (ODC) (Janda and Abbott,
1998), and there have been several reports of fish disease caused by *A. veronii* biovar sobria in Asia and Europe (Rahman et al., 2002; Sreedharan et al., 2011; Smyrli et al., 2017). All present and previous strains in the present study were ADH-positive and ODC-negative in the API 20E test, and were assumed to be *A. veronii* biovar sobria. However, the API profiles of the present strains differed from those of previous strains, and *gyrB* gene sequences from the present strains (LC311630-LC311644) formed a cluster different from other sequences except for the sequence of *A. veronii* isolated from diseased European seabass *Dicentrarchus labrax* in Greece (AERO NS; Smyrli et al., 2017; KF636138) (Fig. 2). Smyrli et al. (2017) reported that mortality of European seabass caused by *A. veronii* was observed in Agean Sea and the Black Sea. These results and information suggest that the *A. veronii* biovar sobria isolated in the present study may have been introduced from other aquatic environments, inside and outside the country.

In our experimental infection by bath exposure, the dead ayu showed clinical signs similar to those observed in naturally infected fish, and *A. veronii* with *gyrB* sequences matching those of the strains used for exposure were isolated from all dead or moribund fish. The cumulative mortalities of ayu exposed to previous strains were 20%-40%, whereas over 80% of fish exposed to the present strains were dead by 3 days post exposure (Fig. 3). These results indicate that the *A. veronii* found

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**Fig. 2.** Phylogenetic tree showing the relationships among *Aeromonas veronii* strains from riverine fish collected in the Tama River Basin. The tree was inferred from *gyrB* gene sequences by the maximum likelihood method. The scale bar represents a 1% sequence difference. Numbers at nodes are bootstrap values (> 50%) after 1,000 iterations. The name of the fish species in parentheses and the shaded boxes show the origins and API 20E profiles of each strain, respectively. *Plecoglossus altivelis, Dicentrarchus labrax, Opsariichthys platypus, Cyprinus carpio.*

**Fig. 3.** Cumulative mortality of ayu challenged by exposure to *Aeromonas veronii* isolated from riverine fish collected in the Tama River Basin compared with that of ayu exposed to a reference strain from the intestine of common carp (CWP11). No dead fish were observed in the control group (exposed with TSB). Solid symbols: “present strains”; hollow symbols: “previous strains”; x: “reference strain.”
in the present study has high pathogenicity to ayu compared to other strains. We conclude that the mass mortalities of riverine ayu found in the tributary of the Tama River in July 2016 were caused by high pathogenic *A. veronii* biovar sobria with different properties than previous strains.

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**References**


多摩川支流域で認められたアユ死亡に関与する病原性
Aeromonas veronii biovar sobria

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柏田祥策・間野伸宏

2016年7月、多摩川支流域において下顎、体表と腹腔
基部の出血および腹水貯留などの症状を呈するアユ死亡
魚が確認された。検査した全ての死亡魚の腎臓からグラ
ム陰性の運動性短桿菌が分離され、Aeromonas veronii
biovar sobria と同定された。今回分離された菌株は、過
去に多摩川生息魚から分離された A. veronii 株と生化学的
性状や gyrB 遺伝子配列が異なっていた。また人間感染
試験において、今回分離された株は過去に分離された株
より高い死亡率を示した。多摩川支流域で認められた今
回のアユの死亡は、過去の分離株とは性状の異なる高病
原性 A. veronii biovar sobria により引き起こされたものと
考えられた。

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